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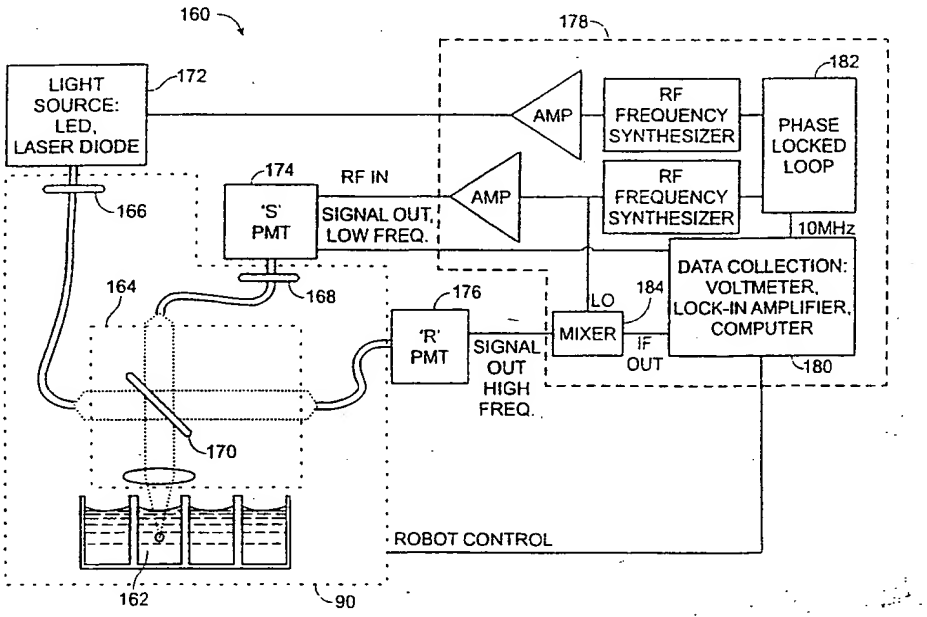
(54) Title: APPARATUS AND METHODS FOR IMPROVING SIGNAL RESOLUTION IN OPTICAL SPECTROSCOPY

(57) Abstract

Apparatus, methods, and compositions of matter for improving signal resolution in optical spectroscopy assays. The apparatus may include components for detecting light emitted by an analyte in a composition. These components may include (1) a stage for supporting the composition, (2) a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte may be induced to emit light, (3) a detector and a second optical relay structure that directs light from the composition toward the detector, so that the light may be detected and converted to a signal, and (4) a processor for analyzing the signal. The processor may be used to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a non–analyte emitter. The non–analyte emitter may include background, and/or the non–analyte emitter may include a reference compound for correcting scattering and absorption, among others.



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APPARATUS AND METHODS FOR IMPROVING SIGNAL RESOLUTION IN OPTICAL SPECTROSCOPY

Cross-References to Related Applications

This application is a continuation of and claims priority from the following patent applications, each of which is incorporated herein by reference: U.S. Patent Application Serial No. 09/062,472, filed April 17, 1998; U.S. Patent Application Serial No. 09/160,533, filed September 24, 1998; and PCT Application Serial No. PCT/US98/23095, filed October 30, 1998.

This application is based upon and claims benefit under 35 U.S.C. § 119 of the following U.S. Provisional Patent Applications, each of which is incorporated herein by reference: Serial No. 60/072,499, filed January 26, 1998; Serial No. 60/072,780, filed January 27, 1998, Serial No. 60/075,806, filed February 24, 1998; and Serial No. 60/084,167, filed May 4, 1998.

This application incorporates by reference the following publication: JOSEPH R. LAKOWICZ, PRINCIPLES OF FLUORESCENCE SPECTROSCOPY (1983).

Field of the Invention

The invention relates to optical spectroscopy. More particularly, the invention relates to apparatus, methods, and compositions of matter for improving signal resolution in optical spectroscopy.

Background of the Invention

Optical spectroscopic assays often use fluorescence and phosphorescence to characterize the components and properties of molecular systems; for example, optical spectroscopic assays may be used in high-throughput screening procedures to identify candidate drug compounds. Optical spectroscopic assays that use fluorescence include fluorescence polarization (FP), fluorescence resonance energy transfer (FRET), fluorescence lifetime (FLT), total internal reflection (TIR) fluorescence, fluorescence correlation spectroscopy (FCS), and fluorescence recovery after photobleaching (FRAP), among others. Optical spectroscopic assays that use phosphorescence include phosphorescence polarization and phosphorescence lifetime, among others. Each assay has strengths and weaknesses. For example, fluorescence and phosphorescence polarization assays are homogeneous and ratiometric, making

them relatively insensitive to sample-to-sample variations in concentration, volume, and meniscus shape.

Optical spectroscopic assays often involve the absorption and subsequent emission of light. Typically, an analyte is excited from its ground state into one of its excited states by the absorption of a photon of light. The excited-state energy associated with this process can be lost through various mechanisms, including production of a photon through fluorescence or phosphorescence, among others. Properties of the fluorescence or phosphorescence may be used to study properties of the analyte and its environment, and to study binding reactions and enzymatic activity involving the analyte, among others.

Optical spectroscopic assays are subject to artifacts that alter the apparent luminescence of the analyte and thus the accuracy, repeatability, and reliability of the assay. Some artifacts increase the apparent luminescence of the analyte, causing intensity-based assays to overreport the amount of light emitted by the analyte. Such artifacts include background. Other artifacts decrease the apparent luminescence of the analyte, causing intensity-based assays to underreport the amount of light emitted by the analyte. Such artifacts include scattering and absorption. Such artifacts also include changes in the composition that change the optical transfer function (photons collected / photons injected), including changes in index of refraction and surface tension.

Optical spectroscopic assays also are subject to artifacts that alter the apparent polarization of the analyte. Such artifacts also include background, scattering, and absorption, among others, and can increase or decrease the apparent polarization.

Among artifacts that alter polarization while increasing the apparent luminescence of the analyte, background is especially significant. Background refers to light and other signals that do not arise from the analyte, but that can be confused with light that does arise from the analyte. Background may arise from non-analyte luminescent components of the sample (e.g., library compounds, target molecules, etc.). Background also may arise from luminescent components of the sample container and detection system (e.g., microplates, optics, fiber optics, etc.). Background also may arise from

scattered excitation light that leaks through the optical filters, which is equivalent to luminescence with a zero lifetime, and from room light.

There is no way to eliminate every source of background, so methods must be used to discriminate between analyte and background. If the analyte and background have different spectra, background may be at least partially discriminated using appropriate optical filters, which pass light emitted by the analyte but block background. If the analyte and background have overlapping spectra, background may be at least partially discriminated in two ways. First, background may be discriminated using a blank. In this method, data such as intensity data are collected for the sample and for a blank that lacks analyte but otherwise resembles the sample. Background is at least partially discriminated by subtracting the data obtained from the blank from the data obtained from the sample. Second, background may be discriminated by gating. In this method, data are collected from the sample only at times when the background is low or nonexistent.

Unfortunately, these methods of rejecting background suffer from a number of shortcomings, especially if the analyte and background have overlapping spectra. The use of blanks requires making two measurements for every sample, at least if the background is different for each sample. Background may be different for each sample if each sample is housed in a different container and/or if each sample contains a different, intrinsically luminescent target molecule, such as a peptide, protein, or nucleic acid, among others. The use of gating requires knowledge of the lifetime and intensity of the background. The use of gating also requires collecting data only over limited times, so that data collection is slowed and potentially useful data is discarded. Gating is especially problematic for short-lifetime background, because luminescence from the analyte is most intense for short times after excitation.

Among artifacts that alter polarization while decreasing the apparent luminescence of the analyte, scattering and absorption are especially significant. Scattering can arise if the composition containing the analyte is turbid, so that excitation and/or emission light are scattered out of the optical path and therefore not detected. Absorption can arise if non-analyte components of the composition can absorb excitation and/or emission light. Absorption of excitation light reduces luminescence indirectly, by reducing the

amount of light available to excite luminescence. Absorption of emission light reduces luminescence directly. Collectively, absorption of excitation and emission light is termed "color quenching." Scattering and color quenching may vary from sample to sample and therefore be difficult to characterize.

There is no way to eliminate every source of scattering and absorption. This is especially true in compositions containing biological molecules, because biological molecules such as proteins and nucleic acids may absorb light having wavelengths commonly used in luminescence assays.

Background, scattering, absorption, and other artifacts affecting apparent luminescence are significant shortcomings, even for single measurements. However, they are potentially crippling shortcomings in high-throughput screening applications, where tens or hundreds of thousands of samples may be analyzed each day. In screening applications, the use of blanks may double the consumption of reagents and the time required for sample preparation and data collection, as well as associated costs. Moreover, in screening applications, biological molecules that scatter and absorb light often must be employed.

Summary of the Invention

The present invention addresses these and other shortcomings by providing apparatus, methods, and compositions of matter for improving signal resolution in optical spectroscopy assays.

The apparatus provided by the invention may include components for detecting light emitted by an analyte in a composition. These components may include (1) a stage, (2) a light source and a first optical relay structure that directs light from the light source toward the composition, (3) a detector and a second optical relay structure that directs light from the composition toward the detector, and (4) a processor. The stage may be used to support the composition. The light source and first optical relay structure may be used to induce the analyte to emit light. The detector and second optical relay structure may be used to detect light transmitted from the composition and to convert the detected light to a signal. The processor may be used to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a non-analyte emitter, signal modifier, or perturbant. The non-analyte emitter may include

background, and/or the non-analyte emitter may include a reference compound for correcting for scattering and absorption, among others.

The processor may employ various algorithms. For example, the processor may discriminate between the first and second portions of the signal without requiring a determination of the lifetime or intensity of the background. The processor also may discriminate between the first and second portions without requiring the use of information obtained from a blank. The processor also may discriminate between the first and second portions in the frequency domain. The processor also may employ other algorithms.

The processor may calculate various quantities. For example, the processor may calculate the intensity of the analyte. The processor also may calculate the polarization of the analyte. The processor also may calculate a quantity that expresses the intensity or polarization of the analyte as a function of the intensity or polarization of a reference compound. The processor also may calculate other quantities.

The methods provided by the invention may include steps for detecting light emitted by an analyte in a composition. These steps may include (1) illuminating the composition, so that light is emitted by the analyte, (2) detecting light transmitted from the composition and converting it to a signal, and (3) processing the signal to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background. The methods also may include additional or alternative steps.

The compositions of matter provided by the invention may include first and second luminophores, wherein the emission spectra of the first and second luminophores overlap significantly, and wherein light emitted by the first luminophore is resolvable from light emitted by the second luminophore using lifetime-resolved methods. The first luminophore may be an analyte, and the second luminophore may be a reference compound.

The nature of the invention will be understood more readily after consideration of the drawings and the detailed description of preferred embodiments that follow.

Brief Description of the Drawings

Figure 1 is a schematic view of a frequency-domain time-resolved measurement, showing the definitions of phase angle (phase) ϕ and demodulation factor (modulation) *M*.

Figure 2 is a schematic view of an apparatus for detecting light emitted by an analyte in a composition in accordance with the invention.

Figure 3 is a partially schematic perspective view of the apparatus of Figure 2.

Figure 4 is a schematic view of optical components from the apparatus of Figure 2.

Figure 5 is a partially exploded perspective view of a housing for the apparatus of Figure 2.

Figure 6 is a schematic view of an alternative apparatus for detecting light emitted by an analyte in a composition in accordance with the invention.

Figure 7 is a phasor diagram showing phase and modulation phasors for a system having an analyte and background.

Figure 8 is a graph of simulation results showing how the invention discriminates between an analyte and background for three zeroth-order embodiments of the invention, as described in Equations 13 (LDI, M_X-based), 15 (LDI, ϕ-based), and 16 (LRI).

Figure 9 is a graph of experimental results showing how the invention discriminates between a long-lifetime ruthenium-complex analyte and a short-lifetime R-phycoerythrin background, for a constant concentration of analyte and an increasing concentration of background. Results are shown for the three embodiments described under Figure 8.

Figure 10 is a graph of experimental results showing how the invention discriminates between a long-lifetime ruthenium-complex analyte and a short-lifetime R-phycoerythrin background, for a constant concentration of background and an increasing concentration of analyte. Results are shown for the three embodiments described under Figure 8.

Figure 11 is a graph of simulation results showing how binding affects phase (Panel A) and modulated anisotropy (Panel B) in the presence of 0% background in a frequency-domain binding experiment, for 0-100% binding as shown.

Figure 12 is a graph of simulation results showing how binding affects phase (Panel A) and modulated anisotropy (Panel B) in the presence of 50% background in the frequency-domain binding experiments shown in Figure 11.

Figure 13 is a graph of simulation results showing how binding affects Ψ'_ω in the presence of 0% (solid lines) and 50% (dashed lines) background in the frequency-domain experiments of Figure 11, for 0-100% binding as shown. Ψ'_ω is defined and evaluated in accordance with the invention.

Figure 14 is a graph of simulation results showing how binding affects K_ω in the presence of 0% (solid lines) and 90% (dashed lines) background in the frequency-domain binding experiments of Figure 11. K_ω is defined and evaluated in accordance with the invention.

Detailed Description of the Invention

The invention provides apparatus, methods, and compositions of matter for improving signal resolution in optical spectroscopy. Generally, the apparatus and methods employ a stage, a light source, a detector, a processor, and first and second optical relay structures for directing light between the light source, composition, and detector. Generally, the compositions of matter include first and second luminophores having emission spectra that overlap significantly, and emissions that are resolvable using lifetime-resolved methods. For clarity, the description of the invention that follows is divided into six parts: (1) overview of luminescence assays, (2) description of luminescence apparatus, (3) intensity assays, (4) polarization assays, (5) reference compounds; and (6) conclusions.

1. Overview of Luminescence Assays

Luminescence assays use luminescence emissions from luminescent analytes to study the properties and environment of the analyte, as well as binding reactions and enzymatic activities involving the analyte, among others. In this sense, the analyte may act as a reporter to provide information about another material or target substance that may be the focus of the assay. Luminescence assays may use various aspects of the luminescence, including its intensity, polarization, and lifetime, among others. Luminescence assays also may use time-independent (steady-state) and/or time-dependent (time-

resolved) properties of the luminescence. Steady-state assays generally are less complicated than time-resolved assays, but generally yield less information.

Intensity Assays. Luminescence intensity assays involve monitoring the intensity (or amount) of light emitted from a composition. The intensity of emitted light will depend on the extinction coefficient, quantum yield, and number of the luminescent analytes in the composition, among others. These quantities, in turn, will depend on the environment on the analyte, among others, including the proximity and efficacy of quenchers and energy transfer partners. Thus, luminescence intensity assays may be used to study binding reactions, among other applications.

Polarization Assays. Luminescence polarization assays involve the absorption and emission of polarized light. (Polarization describes the direction of light's electric field, which generally is perpendicular to the direction of light's propagation.) In a luminescence polarization assay, specific molecules within a composition are labeled with one or more luminophores. The composition then is illuminated with polarized excitation light, which preferentially excites luminophores having absorption dipoles aligned parallel to the polarization of the excitation light. These molecules subsequently decay by preferentially emitting light polarized parallel to their emission dipoles. The extent to which the total emitted light is polarized depends on the extent of molecular reorientation during the time interval between luminescence excitation and emission, which is termed the luminescence lifetime, τ. The extent of molecular reorientation in turn depends on the luminescence lifetime and the size, shape, and environment of the reorienting molecule. Thus, luminescence polarization assays may be used to quantify binding reactions and enzymatic activity, among other applications. In particular, molecules rotate via diffusion with a rotational correlation time τ_{rot} that is proportional to their size. Thus, during their luminescence lifetime, relatively large molecules will not reorient significantly, so that their total luminescence will be relatively polarized. In contrast, during the same time interval, relatively small molecules will reorient significantly, so that their total luminescence will be relatively unpolarized.

The relationship between polarization and intensity is expressed by the following equation:

$$P = \frac{I_{||} - I_{\perp}}{I_{||} + I_{\perp}} \quad (1)$$

Here, P is the polarization, $I_{||}$ is the intensity of luminescence polarized parallel to the polarization of the excitation light, and $I_{\perp}$ is the intensity of luminescence polarized perpendicular to the polarization of the excitation light. If there is little rotation between excitation and emission, $I_{||}$ will be relatively large, $I_{\perp}$ will be relatively small, and P will be close to one. (P may be less than one even if there is no rotation; for example, P will be less than one if the absorption and emission dipoles are not parallel.) In contrast, if there is significant rotation between absorption and emission, $I_{||}$ will be comparable to $I_{\perp}$, and P will be close to zero. Polarization often is reported in milli-P units ($1000 \times P$), which will range between 0 and 1000, because P will range between zero and one.

Polarization also may be described using other equivalent quantities, such as anisotropy. The relationship between anisotropy and intensity is expressed by the following equation:

$$r = \frac{I_{||} - I_{\perp}}{I_{||} + 2I_{\perp}} \quad (2)$$

Here, r is the anisotropy. Polarization and anisotropy include the same information, although anisotropy may be more simply expressed for systems containing more than one luminophore. In the description and claims that follow, these terms may be used interchangeably, and a generic reference to one should be understood to imply a generic reference to the other.

The relationship between polarization and rotation is expressed by the Perrin equation:

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right) \cdot \left(1 + \frac{\tau}{\tau_{rot}}\right) \quad (3)$$

Here, P_0 is the polarization in the absence of molecular motion (intrinsic polarization), τ is the luminescence lifetime (inverse decay rate) as described

above, and τ_{rot} is the rotational correlation time (inverse rotational rate) as described above.

The Perrin equation shows that luminescence polarization assays are most sensitive when the luminescence lifetime and the rotational correlation time are similar. Rotational correlation time is proportional to molecular weight, increasing by about 1 nanosecond for each 2,400 dalton increase in molecular weight (for a spherical molecule). For shorter lifetime luminophores, such as fluorescein, which has a luminescence lifetime of roughly 4 nanoseconds, luminescence polarization assays are most sensitive for molecular weights less than about 40,000 daltons. For longer lifetime probes, such as Ru(bpy)$_2$dcbpy (ruthenium 2,2'-dibipyridyl 4,4'-dicarboxyl-2,2'-bipyridine), which has a lifetime of roughly 400 nanoseconds, luminescence polarization assays are most sensitive for molecular weights between about 70,000 daltons and 4,000,000 daltons.

Time-Resolved Assays. Time-resolved assays involve measuring the time course of luminescence emission. Time-resolved assays may be conducted in the time domain or in the frequency domain, both of which are functionally equivalent. In a time-domain measurement, the time course of luminescence is monitored directly. Typically, a composition containing a luminescent analyte is illuminated using a narrow pulse of light, and the time dependence of the intensity of the resulting luminescence emission is observed, although other protocols also may be used. For a simple molecule, the luminescence commonly follows a single-exponential decay.

In a frequency-domain measurement, the time course of luminescence is monitored indirectly, in frequency space. Typically, the composition is illuminated using light whose intensity is modulated sinusoidally at a single modulation frequency *f*, although other protocols (such as transforming time-domain data into the frequency domain) also may be used. The intensity of the resulting luminescence emission is modulated at the same frequency as the excitation light. However, the emission will lag the excitation by a phase angle (phase) ϕ, and the intensity of the emission will be demodulated relative to the intensity of the excitation by a demodulation factor (modulation) *M*.

Figure 1 shows the relationship between emission and excitation in a single-frequency frequency-domain experiment. The phase ϕ is the phase

difference between the excitation and emission. The modulation M is the ratio of the AC amplitude to the DC amplitude for the emission, relative to the ratio of the AC amplitude to the DC amplitude for the excitation. The phase and modulation are related to the luminescence lifetime τ by Equations 4 and 5.

$$\omega\tau = \tan(\phi) \tag{4}$$

$$\omega\tau = \sqrt{\frac{1}{M^2} - 1} \tag{5}$$

Here ω is the angular modulation frequency, which equals 2π times the modulation frequency. For maximum sensitivity, the angular modulation frequency should be roughly the inverse of the luminescence lifetime. Lifetimes of interest in high-throughput screening vary from less than 1 nanosecond to greater than 10 microseconds. Therefore, instruments for high-throughput screening should be able to cover modulation frequencies from 20 kHz to 200 MHz.

2. Description of Luminescence Apparatus

Figures 2-5 show an apparatus 90 for detecting light emitted by an analyte in a composition. Apparatus 90 may include (1) a stage for supporting the composition, (2) one or more light sources for delivering light to a composition, (3) one or more detectors for receiving light transmitted from the composition and converting it to a signal, (4) first and second optical relay structures for relaying light between the light source, composition, and detector, and (5) a processor for analyzing the signal from the detector. Apparatus 90 may be used for a variety of luminescence and absorbance assays, including but not limited to the assays described above.

As configured here, apparatus 90 includes a continuous light source 100 and a time-modulated light source 102. Apparatus 90 includes light source slots 103a-d for four light sources, although other numbers of light source slots and light sources also could be provided. Light source slots 103a-d function as housings that may surround at least a portion of each light source, providing some protection from radiation and explosion. The direction of light transmission through the photoluminescence optical system is indicated by arrows.

Continuous source 100 provides light for photoluminescence intensity and steady-state photoluminescence polarization assays. Continuous light source 100 may include arc lamps, lasers, laser diodes, and light-emitting diodes (LEDs), among others. A preferred continuous source is a high-intensity, high color temperature xenon arc lamp, such as a Model LX175F CERMAX xenon lamp from ILC Technology, Inc. Color temperature is the absolute temperature in Kelvin at which a blackbody radiator must be operated to have a chromaticity equal to that of the light source. A high color temperature lamp produces more light than a low color temperature lamp, and it may have a maximum output shifted toward or into visible wavelengths and ultraviolet wavelengths where many luminophores absorb. The preferred continuous source has a color temperature of 5600 Kelvin, greatly exceeding the color temperature of about 3000 Kelvin for a tungster filament source. The preferred source provides more light per unit time than flash sources, increasing sensitivity and reducing read times. Apparatus 90 may include a modulator mechanism configured to vary the intensity of light incident on the composition without varying the intensity of light produced by the light source.

Time-modulated source 102 provides light for time-resolved photoluminescence assays, such as photoluminescence lifetime and time-resolved photoluminescence polarization assays. A preferred time-modulated source is a xenon flash lamp, such as a Model FX-1160 xenon flash lamp from EG&G Electro-Optics. The preferred source produces a "flash" of light for a brief interval before signal detection and is especially well suited for time-domain measurements. Other time-modulated sources include pulsed lasers, as well as continuous lamps whose intensity can be modulated extrinsically using a Pockels cell, Kerr cell, or other mechanism. The latter sources are especially well suited for frequency-domain measurements.

In apparatus 90, continuous source 100 and time-modulated source 102 produce multichromatic, unpolarized, and incoherent light. Continuous source 100 produces substantially continuous illumination, whereas time-modulated source 102 produces time-modulated illumination. Light from these light sources may be delivered to the sample without modification, or it may be filtered to alter its intensity, spectrum, polarization, or other properties.

Light produced by the light sources follows an excitation optical path to an examination site. Such light may pass through one or more "spectral filters," which generally comprise any mechanism for altering the spectrum of light that is delivered to the sample. Spectrum refers to the wavelength composition of light. A spectral filter may be used to convert white or multichromatic light, which includes light of many colors, into red, blue, green, or other substantially monochromatic light, which includes light of one or only a few colors. In apparatus 90, spectrum is altered by an excitation interference filter 104, which selectively transmits light of preselected wavelengths and selectively absorbs light of other wavelengths. For convenience, excitation interference filters 104 may be housed in an excitation filter wheel 106, which allows the spectrum of excitation light to be changed by rotating a preselected filter into the optical path. Spectral filters also may separate light spatially by wavelength. Examples include gratings, monochromators, and prisms.

Spectral filters are not required for monochromatic ("single color") light sources, such as certain lasers, which output light of only a single wavelength. Therefore, excitation filter wheel 106 may be mounted in the optical path of some light source slots 103a,b, but not other light source slots 103c,d.

Light next passes through an excitation optical shuttle (or switch) 108, which positions an excitation fiber optic cable 110a,b in front of the appropriate light source to deliver light to top or bottom optics heads 112a,b, respectively. The optics heads include various optics for delivering light into the sensed volume and for receiving light transmitted from the sensed volume. Light is transmitted through a fiber optic cable much like water is transmitted through a garden hose. Fiber optic cables can be used easily to turn light around corners and to route light around opaque components of the apparatus. Moreover, fiber optic cables give the light a more uniform intensity profile. A preferred fiber optic cable is a fused silicon bundle, which has low autoluminescence. Despite these advantages, light also can be delivered to the optics heads using other mechanisms, such as mirrors.

Light arriving at the optics head may pass through one or more excitation "polarization filters," which generally comprise any mechanism for altering the polarization of light. Excitation polarization filters may be included with the top and/or bottom optics head. In apparatus 90, polarization is altered

by excitation polarizers 114, which are included only with top optics head 112a. Excitation polarization filters 114 may include an s-polarizer S that passes only s-polarized light, a p-polarizer P that passes only p-polarized light, and a blank O that passes substantially all light. Excitation polarizers 114 also may include a standard or ferro-electric liquid crystal display (LCD) polarization switching system. Such a system is faster and more economical than a mechanical switcher. Excitation polarizers 114 also may include a continuous mode LCD polarization rotator with synchronous detection to increase the signal-to-noise ratio in polarization assays. Excitation polarizers 114 may be included in light sources, such as certain lasers, that intrinsically produce polarized light.

Light at one or both optics heads also may pass through an excitation "confocal optics element," which generally comprises any mechanism for focusing light into a "sensed volume." In apparatus 90, the confocal optics element includes a set of lenses 117a-c and an excitation aperture 116 placed in an image plane conjugate to the sensed volume, as shown in Figure 4. Aperture 116 may be implemented directly, as an aperture, or indirectly, as the end of a fiber optic cable. Lenses 117a,b project an image of aperture 116 onto the sample, so that only a preselected or sensed volume of the sample is illuminated.

Light traveling through the optics heads is reflected and transmitted through a beamsplitter 118, which delivers reflected light to a composition 120 and transmitted light to a light monitor 122. Reflected and transmitted light both pass through lens 117b, which is operatively positioned between beamsplitter 118 and composition 120.

The beamsplitter is used to direct excitation light toward the sample and light monitor, and to direct emission light toward the detector. The beamsplitter is changeable, so that it may be optimized for different assay modes or compositions. If a large number or variety of luminescent molecules are to be studied, the beamsplitter must be able to accommodate light of many wavelengths; in this case, a "50:50" beamsplitter that reflects half and transmits half of the incident light independent of wavelength is optimal. Such a beamsplitter can be used with many types of molecules, while still delivering considerable excitation light onto the composition, and while still transmitting considerable emission light to the detector. If one or a few related luminescent

molecules are to be studied, the beamsplitter needs only to be able to accommodate light at a limited number of wavelengths; in this case, a "dichroic" or "multichroic" beamsplitter is optimal. Such a beamsplitter can be designed with cutoff wavelengths for the appropriate set of molecules and will reflect most or substantially all of the excitation and background light, while transmitting most or substantially all of the emission light. This is possible because the reflectivity and transmissivity of the beamsplitter can be varied with wavelength.

The light monitor is used to correct for fluctuations in the intensity of light provided by the light sources; such corrections are performed by reporting detected intensities as a ratio over corresponding times of the luminescence intensity measured by the detector to the excitation light intensity measured by the light monitor. The light monitor also can be programmed to alert the user if the light source fails. A preferred light monitor is a silicon photodiode with a quartz window for low autoluminescence.

The composition (or sample) may be held in a sample holder supported by a stage 123. The composition can include compounds, mixtures, surfaces, solutions, emulsions, suspensions, cell cultures, fermentation cultures, cells, tissues, secretions, and/or derivatives and/or extracts thereof. Analysis of the composition may involve measuring the presence, concentration, or physical properties (including interactions) of a photoluminescent analyte in such a composition. The sample holder can include microplates, biochip, or any array of samples in a known format. In apparatus 90, the preferred sample holder is a microplate 124, which includes a plurality of microplate wells 126 for holding compositions. Composition may refer to the contents of a single microplate well, or several microplate wells, depending on the assay. In some embodiments, such as a portable analyzer, the stage may be intrinsic to the instrument.

The sensed volume typically has an hourglass shape, with a cone angle of about 25° and a minimum diameter ranging between 0.1 mm and 2.0 mm. For 96-well and 384-well microplates, a preferred minimum diameter is about 1.5 mm. For 1536-well microplates, a preferred minimum diameter is about 1.0 mm. The size and shape of the sample container may be matched to the size and shape of the sensed volume.

The position of the sensed volume can be moved precisely within the composition to optimize the signal-to-noise and signal-to-background ratios. For example, the sensed volume may be moved away from walls in the sample holder to optimize signal-to-noise and signal-to-background ratios, reducing spurious signals that might arise from luminophore bound to the walls and thereby immobilized. In apparatus 90, position in the X,Y-plane perpendicular to the optical path is controlled by moving the stage supporting the composition, whereas position along the Z-axis parallel to the optical path is controlled by moving the optics heads using a Z-axis adjustment mechanism 130, as shown in Figures 2 and 3. However, any mechanism for bringing the sensed volume into register or alignment with the appropriate portion of the composition also may be employed.

The combination of top and bottom optics permits assays to combine: (1) top illumination and top detection, or (2) top illumination and bottom detection, or (3) bottom illumination and top detection, or (4) bottom illumination and bottom detection. Same-side illumination and detection (1) and (4) is referred to as "epi" and is preferred for photoluminescence assays. Opposite-side illumination and detection (2) and (3) is referred to as "trans" and is preferred for absorbance assays. In apparatus 90, epi modes are supported, so the excitation and emission light travel the same path in the optics head, albeit in opposite or anti-parallel directions. However, trans modes also could be supported and would be essential for absorbance assays. Generally, top optics can be used with any sample holder having an open top, whereas bottom optics can be used only with sample holders having optically transparent bottoms, such as glass or thin plastic bottoms.

Light is transmitted by the composition in multiple directions. A portion of the transmitted light will follow an emission pathway to a detector. Transmitted light passes through lens 117c and may pass through an emission aperture 131 and/or an emission polarizer 132. In apparatus 90, the emission aperture is placed in an image plane conjugate to the sensed volume and transmits light substantially exclusively from this sensed volume. In apparatus 90, the emission apertures in the top and bottom optical systems are the same size as the associated excitation apertures, although other sizes also may be used. The emission polarizers are included only with top optics head 112a. The

emission aperture and emission polarizer are substantially similar to their excitation counterparts. Emission polarizer 132 may be included in detectors that intrinsically detect the polarization of light.

Excitation polarizers 114 and emission polarizers 132 may be used together in nonpolarization assays to reject certain background signals. Luminescence from the sample holder and from luminescent molecules adhered to the sample holder is expected to be polarized, because the rotational mobility of these molecules should be hindered. Such polarized background signals can be eliminated by "crossing" the excitation and emission polarizers, that is, setting the angle between their transmission axes at 90°. As described above, such polarized background signals also can be reduced by moving the sensed volume away from walls of the sample holder. To increase signal level, beamsplitter 118 should be optimized for reflection of one polarization and transmission of the other polarization. This method will work best where the luminescent molecules of interest emit relatively unpolarized light, as will be true for small luminescent molecules in solution.

Transmitted light next passes through an emission fiber optic cable 134a,b to an emission optical shuttle (or switch) 136. This shuttle positions the appropriate emission fiber optic cable in front of the appropriate detector. In apparatus 90, these components are substantially similar to their excitation counterparts, although other mechanisms also could be employed.

Light exiting the fiber optic cable next may pass through one or more emission "intensity filters," which generally comprise any mechanism for reducing the intensity of light. Intensity refers to the amount of light per unit area per unit time. In apparatus 90, intensity is altered by emission neutral density filters 138, which absorb light substantially independent of its wavelength, dissipating the absorbed energy as heat. Emission neutral density filters 138 may include a high-density filter H that absorbs most incident light, a medium-density filter M that absorbs somewhat less incident light, and a blank O that absorbs substantially no incident light. These filters are changed by hand, although other methods also could be employed, such as a filter wheel. Intensity filters also may divert a portion of the light away from the sample without absorption. Examples include beam splitters, which transmit some light

along one path and reflect other light along another path, and Pockels cells, which deflect light along different paths through diffraction.

Light next may pass through an emission interference filter 140, which may be housed in an emission filter wheel 142. In apparatus 90, these components are substantially similar to their excitation counterparts, although other mechanisms also could be employed. Emission interference filters block stray excitation light, which may enter the emission path through various mechanisms, including reflection and scattering. If unblocked, such stray excitation light could be detected and misidentified as photoluminescence, decreasing the signal-to-background ratio. Emission interference filters can separate photoluminescence from excitation light because photoluminescence has longer wavelengths than the associated excitation light. Luminescence typically has wavelengths between 200 and 2000 nanometers.

The relative positions of the spectral, intensity, polarization, and other filters presented in this description may be varied without departing from the spirit of the invention. For example, filters used here in only one optical path, such as intensity filters, also may be used in other optical paths. In addition, filters used here in only top or bottom optics, such as polarization filters, may also be used in the other of top or bottom optics or in both top and bottom optics. The optimal positions and combinations of filters for a particular experiment will depend on the assay mode and the composition, among other factors.

Light last passes to a detector, which is used in absorbance and photoluminescence assays. In apparatus 90, there is one photoluminescence detector 144, which detects light from all photoluminescence modes. A preferred detector is a photomultiplier tube (PMT). Apparatus 90 includes detector slots 145a-d for four detectors, although other numbers of detector slots and detectors also could be provided.

More generally, detectors comprise any mechanism capable of converting energy from detected light into signals that may be processed by the apparatus, and by the processor in particular. Suitable detectors include photomultiplier tubes, photodiodes, avalanche photodiodes, charge-coupled devices (CCDs), and intensified CCDs, among others. Depending on the

detector and assay mode. such detectors may be used in (1) photon-counting or continuous modes, and (2) imaging or integrating modes.

Figure 5 shows a housing 150 and other accessories for the apparatus of Figures 2-4. Housing 150 substantially encloses the apparatus, forming (together with light source slots 103a-d) two protective layers around the continuous high color temperature xenon arc lamp. Housing 150 permits automated sample loading and switching among light sources and detectors, further protecting the operator from the xenon arc lamp and other components of the system.

Figure 6 shows an alternative apparatus 160 for detecting light emitted by an analyte in a composition 162. Apparatus 160 includes substantial portions of apparatus 90, including its fiber-optic-coupled optics head 164, excitation 166 and emission 168 filters, dichroic beam splitter 170, and mechanisms for sample positioning and focus control. However, apparatus 160 also may include alternative light sources 172, alternative sample ('S') 174 and reference ('R') 176 detectors, and alternative detection electronics 178. In Figure 6, alternative components 172-178 are shown outside apparatus 90, but they readily may be included inside housing 150 of apparatus 90, if desired.

Apparatus 160 may excite luminescence in various ways, such as using an LED or laser diode light source. For example, analytes absorbing blue light may be excited using a NICHIA-brand bright-blue LED (Model Number NSPB500; Mountville, PA). This LED produces broad-spectrum excitation light, so excitation filter 166 typically is used to block the red edge of the spectrum. If analytes are excited using a laser diode, an excitation filter is not necessary.

Apparatus 160 may detect luminescence and convert it to a signal in various ways. Luminescence can be detected using sample PMT 174, which may be an ISS-brand gain-modulated PMT (Champaign, IL). High-frequency luminescence can be frequency down-converted to a low-frequency signal using a technique called heterodyning. The phase and modulation of the low-frequency signal can be determined using a lock-in amplifier 180, such as a STANFORD RESEARCH SYSTEMS brand lock-in amplifier (Model Number SR830; Sunnyvale, CA). Lock-in amplifier 180 is phase locked using a phase-locked loop 182 to the modulation frequency of light source 172. To correct for

drift in the light source. the output of light source 172 may be monitored using reference PMT 176. which may be a HAMAMATSU-brand PMT (Model Number H6780; Bridgewater, NJ). If reference PMT 176 can respond to high-frequency signals. the heterodyning step can be performed using an external mixer 184. The phase and modulation of reference PMT 176 also may be captured by lock-in amplifier 180 and used to normalize the signal from sample PMT 174.

A computer or processor controls the apparatus, including the external components. The computer also directs sample handling and data collection. Generally, phase and modulation data are collected at one or more frequencies appropriate for the lifetime of the analyte. For background-rejection modes, such as those described below, phase and modulation are measured at one or a few frequencies and processed by the computer or processor to calculate quantities such as the lifetime-discriminated intensity.

Apparatus 90 and apparatus 160 both may be used to conduct a variety of steady-state and time-resolved luminescence assays. Steady-state assays measure luminescence under constant illumination, using the continuous light source. Time-resolved polarization assays measure luminescence as a function of time, using either the continuous light source, with its intensity appropriately modulated, or the time-varying light source.

Intensity assays may be conducted by monitoring the intensity of the luminescence emitted by the composition.

Polarization assays may be conducted as follows. Excitation light from the continuous light source is directed through an excitation filter, low-luminescence fiber optic cable, and excitation polarization filter. Excitation light then is directed to a beamsplitter, which reflects most of the light onto a composition and transmits a little of the light into a light monitor. Emitted light from the composition is directed back through the beamsplitter and then is directed through another low-luminescence fiber optic cable, an emission filter, and a polarization filter (in either the S or P orientation) before detection by a photomultiplier tube. Two measurements are performed for each composition, one with excitation and emission polarizers aligned and one with excitation and emission polarizers crossed. Either polarizer may be static or dynamic, and

either polarizer may be set in the S or P orientation, although typically the excitation polarizer is set in the S orientation.

Additional luminescence assays, including FRET, FLT, TIR, FCS, and FRAP, as well as their phosphorescence analogs, may be conducted using procedures outlined in PRINCIPLES OF FLUORESCENCE SPECTROSCOPY and generally known to persons of ordinary skill in the art.

3. Intensity Assays

The apparatus and methods provided by the invention can be used to discriminate between analyte and background in intensity assays. Background-corrected intensities derived from such intensity assays can be used directly, as intensities, or they can be used indirectly to determine quantities such as polarization and luminescence lifetime. Generally, the invention permits determination of background-corrected intensities for systems having one or more analytes and one or more background components.

Two-component analysis. In systems having two detectable components, such as analyte and background, the contribution of each component to the total intensity can be determined using the intensity, phase, and modulation of the system, measured at a single angular modulator frequency ω. This embodiment of the invention may be termed lifetime-discriminated intensity (LDI).

In the time domain, the luminescence of a complex luminophore or of a mixture of luminophores normally decays as a series of exponentials.

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \qquad (6)$$

Here, $I(t)$ is the time-dependent luminescence intensity, α_i is a preexponential factor, and τ_i is the luminescence lifetime of the ith component. The fraction of the steady-state luminescence intensity contributed by each component may be found by integrating Equation 6 over time.

$$f_i = \alpha_i \tau_i \Big/ \sum_j \alpha_j \tau_j \qquad (7)$$

Here, f_i is the fractional intensity of the ith component.

In the frequency domain, the phase and modulation phasor of a complex luminophore or a mixture of luminophores is a vector sum of the phase and

modulation of the individual components, weighted by the individual components' fractional contributions to the total intensity.

Figure 7 shows phase and modulation for a system containing two luminophores, such as an analyte and background. The phase and modulation of the system can be expressed in terms of X and Y components of the phasor.

$$M_s = \sqrt{M_{s,x}^{\ 2} + M_{s,y}^{\ 2}} \tag{8}$$

$$\phi_s = \arctan\left(\frac{M_{s,y}}{M_{s,x}}\right) \tag{9}$$

Here '*s*' denotes system, and '*x*' and '*y*' denote X and Y components. The X and Y components for the system can be expressed in terms of X and Y components for the analyte and background alone.

$$M_{s,x} \equiv M_s \cdot \cos\phi_s = f_a \cdot M_a \cdot \cos\phi_a + (1-f_a) \cdot M_b \cdot \cos\phi_b \tag{10}$$

$$M_{s,y} \equiv M_s \cdot \sin\phi_s = f_a \cdot M_a \cdot \sin\phi_a + (1-f_a) \cdot M_b \cdot \sin\phi_b \tag{11}$$

Here '*a*' denotes analyte, and '*b*' denotes background.

Equations 10 and 11 can be rearranged to solve for the fractional intensities of the analyte and background. The fractional intensity f_a of the analyte is

$$f_a = \frac{M_{b,i} - M_{s,i}}{M_{b,i} - M_{a,i}} \tag{12}$$

Here '*i*' denotes *x* or *y*, corresponding to X or Y components. To calculate fractional intensity using Equation 7, three quantities must be known: $M_{s,x}$, corresponding to the system; $M_{a,x}$, corresponding to analyte alone; and $M_{b,x}$, corresponding to background alone. $M_{s,x}$ is determined for each sample, by making a measurement on each sample. $M_{a,x}$ is determined for each analyte, not for each sample, either (1) by measuring the modulation using a blank containing the analyte "without" background (possibly at high concentration), or (2) by calculating the modulation using Equations 4-5 and the analyte lifetime as measured above without background. The assumption is that the analyte is the same in every sample but that the background is different in every sample. $M_{b,x}$ is estimated for each sample, by making a measurement on a blank for each sample. $M_{b,x}$ typically varies from sample to sample, because the

background may include contributions both from the composition and from the holder and optics surrounding the composition. $M_{b,x}$ may be estimated by making a measurement on a blank and assuming that the measured value can be applied to each sample.

The apparatus and methods provided by the invention allow a more elegant and accurate solution to background correction, which does not require the use of a blank. Equation 10 can be rewritten as a power series of $\omega\tau_b$ if the background has a short lifetime ($\omega\tau_b<<1$), and as a power series of $1/\omega\tau_b$ if the background has a long lifetime ($1/\omega\tau_b<<1$). If the background has a short lifetime, the analyte fractional intensity is

$$f_a = \frac{1-M_{s,x}}{1-M_{a,x}} + \frac{M_{a,x}-M_{s,x}}{(1-M_{a,x})^2}\cdot(\omega\tau_b)^2 + \cdots \xrightarrow[\omega\tau_b\to 0]{\lim} \frac{1-M_{s,x}}{1-M_{a,x}} \qquad (13)$$

If the background has a long lifetime, the analyte fractional intensity is

$$f_a = \frac{M_{s,x}}{M_{a,x}} + \frac{M_{s,x}-M_{a,x}}{M_{a,x}^{\,2}}\cdot\frac{1}{(\omega\tau_b)^2} + \cdots \xrightarrow[\omega\tau_b\to\infty]{\lim} \frac{M_{s,x}}{M_{a,x}} \qquad (14)$$

Equations 12 and 13 discriminate between light emitted by the analyte and short- or long-lifetime background, based on differences in lifetime, without requiring the lifetime or intensity of the background. If the value of the background lifetime is unknown, Equation 13 can be evaluated by setting $\omega\tau_b$ equal to zero (short lifetimes), and Equation 14 can be evaluated by setting $\omega\tau_b$ equal to infinity (long lifetimes). If the value of the background lifetime is known, Equations 13 and 14 can be evaluated exactly, yielding an improved value of f_a.

Equations 8, 9, and 11 also can be rewritten as power series. In this way, various equations can be derived. For example, in a phase-based formulation, if the background has a short lifetime, the analyte fractional intensity is

$$f_a = \frac{\tan\phi_s}{M_{a,y}+(1-M_{a,x})\cdot\tan\phi_s} + \frac{M_{a,y}+(2-M_{a,x})\cdot\tan\phi_s}{(M_{a,y}+(1-M_{a,x})\cdot\tan\phi_s)^2}\cdot\omega\tau_b \qquad (15)$$

Variations in the intensity and lifetime of the background do not affect the determination of f_a, as long as $\omega\tau_b$ is small (Equations 13 and 15) or large

(Equation 14). This is true even if the background includes multiple components, as long as the lifetime of each component is short (Equations 13 and 15) or long (Equation 14). In this case, it is appropriate to use the average or effective lifetime of the background in evaluating Equations 13-15.

Various factors determine which equation is best for discriminating between analyte and background. One factor is background lifetime: if background lifetime is short, an equation expanded in $\omega\tau_b$ should be used; if background lifetime is long, an equation expanded in $1/\omega\tau_b$ should be used. Another factor is knowledge of background lifetime: if τ_b is unknown, Equations 13 and 14 generally should be used, because they are second (and higher) order in lifetime and so relatively insensitive to the value of the lifetime. Yet another factor is experimental: to avoid recording average intensity and to permit detection electronics to be optimized for AC operation, Equation 15 generally should be used. Yet another factor is the nature of the background: if the background has both short- and long-lifetime components, Equations 13 and 15 will overestimate analyte intensity, because long-lifetime background will be confused with analyte, and Equation 14 will underestimate analyte intensity, because short-lifetime background will be confused with analyte. In such situations, a three-component analysis should be used.

Three-component analysis. In a system having three detectable components, such as an analyte and both short- and long-lifetime backgrounds, the contribution of each component to the total intensity can be determined using the intensity, phase, and modulation of the system, measured at two angular modulation frequencies (ω_1,ω_2). In this case, the fractional intensity of the analyte is

$$f_a = p(\omega_1) - q(\omega_1)\cdot\frac{p(\omega_2)-p(\omega_1)}{q(\omega_2)-q(\omega_1)} \tag{15}$$

$$p(\omega) \equiv \frac{1-M_{s,x}}{1-M_{a,x}} + \frac{M_{a,x}-M_{s,x}}{(1-M_{a,x})^2}\cdot(\omega\tau_{hs})^2 \tag{16}$$

$$q(\omega) \equiv \frac{1/(\omega\tau_{hl})^2 - 1}{1-M_{a,x}} + \frac{1/(\omega\tau_{hl})^2 - M_{a,x}}{(1-M_{a,x})^2}\cdot(\omega\tau_{hs})^2 \tag{17}$$

Here '*bs*' and '*bl*' denote short- and long-lifetime background, respectively. Equations 17 and 18 are correct to third order for single exponential backgrounds. If the short- and/or long-lifetime background include multiple components, the average or effective lifetime of the short components and the average or effective lifetime of the long components should be used for τ_{bs} and τ_{bl}, respectively. This embodiment of the invention may be termed lifetime-resolved intensity (LRI).

Practical considerations. Figure 8 shows simulation results demonstrating the ability of the invention to discriminate between an analyte and a background. Results are shown for three zeroth-order embodiments of the invention, as described in Equations 13 (LDI, M_X-based), 15 (LDI, ϕ-based), and 16 (LRI). The error is determined by the choice of frequency and analyte lifetime. When the lifetimes of the analyte and background differ by more than a factor of ten for the equations based on the X components of the modulation, the error is low enough (<2%) for HTS applications.

The choice of frequency also is important for small systematic errors. In the lifetime-discriminated case (Equation 13), the frequency must be chosen so that the measured quantity ($M_{s,x}$) is useable. The errors in $M_{s,x}$ must not translate into a large uncertainty in the derived fractional intensity. If the fraction of analyte is large, any frequency appropriate for measuring the analyte will suffice. For example, if the analyte has a lifetime of 100 nanoseconds, any frequency in the range of 300 kHz to 8 MHz is appropriate (from 1/5 to 5× the inverse lifetime).

If the fraction of analyte is low, however, the frequency selection is constrained by the fact that $M_{s,x}$ is dominated by the short lifetime background. Its value will be too close to the upper limit (1.000) if the frequency is too small. A normal value for the error in M would be 0.005. With this size error, it is not reasonable to make a precise measurement of M when its value is greater than 0.980. This upper limit will make low frequencies unusable. For a ruthenium-complex analyte having a lifetime of 360 nanoseconds and a background having a lifetime of <5 nanoseconds, a reasonable frequency is 2-3 MHz.

In the lifetime-resolved case (Equation 16), the choice of frequencies is more difficult. Roughly, one frequency is needed to discriminate between the long and intermediate lifetimes, and one frequency is needed to discriminate between the intermediate and short lifetimes. Each frequency may be chosen as for a two-component system. However, using an optimization program to choose the frequencies may be more reliable and robust. The program optimizes the frequencies to minimize systematic error due to finite lifetimes of the short and long components, while also minimizing the error due to changes in analyte lifetime.

Experimental verification. The luminescence intensity due to the analyte can be found by multiplying the total intensity by the calculated fractional intensity, using Equations 12 (LDI, M_X-based), 14 (LDI, ϕ-based), or Equation 15 (LRI), among others. Total intensity is obtained from the steady-state value of the luminescence emission, without performing a separate experiment. To test these concepts, we built a phase and modulation fluorometer capable of measuring samples in a microplate, as described above. The instrument uses epi-luminescence geometry, an intensity-modulated blue LED, and a gain-modulated PMT.

Experiments were conducted to assess the ability of the apparatus and methods to discriminate between analyte and background. The analyte was $[Ru(bpy)_3]Cl_2$ (ruthenium tris-2,2'-bipyridyl chloride), which has a long lifetime in buffer (measured at 330 nanoseconds at a temperature of 26-28 °C in 20 millimolar PBS, pH 7.4). The background was from the sample container and/or added R-phycoerythrin. R-phycoerythrin was used as an intentional background contaminant because its excitation and emission spectra overlap those of $Ru(bpy)_3$ and because it has a short lifetime in buffer (measured at 2.9 nanoseconds in 20 millimolar PBS, pH 7.4). All samples were prepared with 20 mM PBS, pH 7.4, and all data were collected with a 400 millosecond integration time in COSTAR-brand flat-black 96-well microplates.

Ruthenium is a good long-lifetime probe for several reasons. First, ruthenium has a long lifetime. Second, ruthenium's lifetime is not extremely sensitive to oxygen concentration, even though ruthenium sometimes is used as an oxygen sensor. This is because ruthenium's lifetime is short relative to good oxygen sensors. In particular, ruthenium's lifetime is not particularly sensitive

to normal changes in oxygen content in air-equilibrated buffer, so that no special measures must be taken to remove oxygen from the system. Third, ruthenium is an atomic luminophore, so that it is not subject to the common problem of photobleaching. Finally, the ruthenium complex has a convenient excitation spectrum (460 nanometer peak) and a large (140 nanometer) Stokes' shift. (The Stokes' shift is the separation between maxima in excitation and emission spectra.)

Conventional background subtraction fails when the background concentration is too large due to fluctuations in background intensity and variations from sample to sample. A 1% variation between samples will make it impossible to measure an analyte whose intensity is only 1% of the background signal. To have confidence that a signal exists, a three standard-deviations rule may be used. The minimum resolvable signal is defined as a signal that is three standard deviations larger than the average background. For a background-subtracted value, our confidence limit translates to a fractional error (or coefficient of variation, CV) of about 47%. (Both sample and background were assumed to have the same error with the difference three times the error; $CV=\sqrt{3}/2$.) Such a large CV is usable only for qualitative measurements. For quantitative measurements, a smaller CV is desired. Typical dispensing errors, concentration errors, and instrument drift can combine to give an error of several percent. Considering these other errors, it is practical to use data with a 10% CV for quantitative work, which may be considered the limit for precise data. These confidence and precision limits allow quantitatively comparison of data from background-subtracted intensity, lifetime-discriminated intensity, and lifetime-resolved intensity measurements.

Figure 9 shows experimental results demonstrating sensitivity to background, determined by adding increasing concentrations of R-phycoerythrin to a constant concentration of $Ru(bpy)_3$. The result was a series of solutions with increasing total intensity but constant analyte intensity. All solutions were prepared in duplicate, and errors in the average were compared with expected values. Figure 9 shows three curves. LDI corresponds to Equation 13, evaluated at 2.85 MHz. LRI corresponds to Equation 16, evaluated at f_1 = 0.35 MHz and f_2 = 4.33 MHz. BSI corresponds to the

background-subtracted intensity, computed using a blank. The ability of a method to discriminate analyte and background is given by the analyte fractional intensity at which measurement error exceeds the confidence limit. The background-subtraction method can discriminate between analyte and background only if the analyte fractional intensity exceeds 17%, whereas LDI and LRI can discriminate between analyte and background if the analyte fractional intensity exceeds 2% and < 0.8%, respectively. Therefore, both methods are less than one-tenth as responsive to background luminescence as background subtraction. This reduced responsivity is achieved while reducing experimental complexity. Under the proper conditions, LDI and LRI do not require any measurement of the background luminescence, including its lifetime and intensity. The contribution of background to the measured intensity is removed simply because of its short lifetime.

Figure 10 shows experimental results demonstrating sensitivity to analyte, determined by adding increasing concentrations of $Ru(bpy)_3$ to a constant (1 nanomolar) concentration of R-phycoerythrin. The result was a series of solutions with increasing total intensity but constant background intensity. This setup permits a determination of the minimum resolvable fraction of analyte in the presence of background. All solutions were prepared in duplicate, and errors in the average were compared with expected values. We measured the LDI was measured at 2.85 MHz, and LRI was measured at 0.35 and 2.85 MHz. The difference between methods is again substantial. Background subtraction quickly fails to resolve the analyte (at a fractional intensity of 13% or 100 micromolar of ruthenium complex). LDI reports the correct analyte intensity down to a fractional intensity of 1% (10 μM), while LRI reports the correct intensity down to less than 0.7% (5 micromolar). This is a greater than tenfold increase in the sensitivity to the analyte for either method. These consistent results suggest that LDI and LRI measurements can be a significant improvement over conventional background subtraction.

The invention is robust, simple, and fast, making it ideal for high-throughput screening. LDI is able accurately to distinguish short- and long-lifetime components using phase and modulation at only a single frequency. LRI is able accurately to separate three lifetime components using phase and modulation at two frequencies. Extension to even more components also is

possible. Knowledge of the lifetime of one component is used to determine the intensity of each component, without requiring a determination of the lifetime or intensity of the other component.

4. Polarization Assays

The apparatus and methods provided by the invention also can be used to discriminate between analyte and background in polarization assays. Generally, the invention permits determination of background-corrected polarizations for systems having one or more analytes and one or more background components.

Background-corrected steady-state polarizations (or anisotropies) may be determined using Equation 1, where $I_{||}$ and $I_{\perp}$ may be determined using appropriate combinations of parallel and perpendicular excitation and emission polarizers, and the apparatus and methods described above for computing background-corrected intensities. Such corrections are important, because steady-state anisotropies are intensity-weighted averages of the anisotropies of all components present, so that background affects the measured anisotropies directly.

Background-corrected time-resolved polarizations (or anisotropies) may be determined using time-domain or frequency-domain techniques. In the time domain, background-corrected polarizations may be determined using Equation 1, where $I_{||}$ and $I_{\perp}$ are replaced by $I_{||}(t)$ and $I_{\perp}(t)$. In the frequency domain, background-corrected polarizations may be determined using appropriate combinations of parallel and perpendicular phase ϕ_p and parallel and perpendicular modulation M_p. Here 'p' denotes parallel or perpendicular, corresponding to parallel and perpendicular components. ϕ_p and M_p are determined using the same apparatus and methods as ϕ and M, with the addition of parallel and perpendicular polarizers, as appropriate. ϕ_p and M_p may be rewritten in terms of ω and I(t).

$$\phi_{p\omega} = \tan^{-1}(N_{p\omega} / D_{p\omega}) \tag{19}$$

$$M_{p\omega} = \sqrt{N_{p\omega}^2 + D_{p\omega}^2} / J_p \tag{20}$$

$$J_p = \int_0^\infty I_p(t)dt \tag{21}$$

$$N_{p\omega} = \int_0^\infty I_p(t)\sin(\omega t)dt \tag{22}$$

$$D_{p\omega} = \int_0^\infty I_p(t)\cos(\omega t)dt \tag{23}$$

Experimental results may be interpreted using a differential phase angle Δ_ω and a ratio Λ_ω of the parallel and perpendicular AC components of the polarized emission.

$$\Delta_\omega = \phi_{\perp\omega} - \phi_{\parallel\omega} \tag{24}$$

$$\Lambda_\omega = \frac{AC_\parallel}{AC_\perp} = \frac{\sqrt{N_{\parallel\omega}^2 + D_{\parallel\omega}^2}}{\sqrt{N_{\perp\omega}^2 + D_{\perp\omega}^2}} \tag{25}$$

Λ_ω may be used to define a frequency-dependent quantity r_ω, called the modulated anisotropy.

$$r_\omega = \frac{\Lambda_\omega - 1}{\Lambda_\omega + 2} \tag{26}$$

r_ω tends to the fundamental anisotropy r_0 at high frequency and to the steady-state anisotropy r_{ss} at low frequency.

Frequency-domain time-resolved polarization may be used to investigate the motional properties of biological molecules in more detail than steady-state polarization. For example, a biophysical model may be used to generate functional forms of $I_\parallel(t)$ and $I_\perp(t)$, using parameters such as lifetimes and rotational correlation times. This model can be used to predict Δ_ω and Λ_ω. Experiments then can be done to measure Δ_ω and Λ_ω, at one or more modulation frequencies. Experimental results may be fitted to the model by adjusting the parameters to give the best fit between predicted and observed values of Δ_ω and Λ_ω or r_ω, for example, by using nonlinear least-squares optimization algorithms.

Alternatively, a simpler approach may be used, in which experiments are conducted at one or a few modulation frequencies, and experimental results are interpreted without resort to fitting to detailed models. Such an approach may be sufficient quickly to assay for significant changes in molecular mobility, for

example, as occurs upon binding. Such binding may be to a target molecule as part of an assay, or to walls of the sample container, among others.

Figure 11 shows how Δ_ω (Panel A) and r_ω (Panel B) depend on ω for a simple binding system in the absence of background. Here, the labeled molecule has a fundamental anisotropy r_0=0.3, a luminescence lifetime τ=100 nanoseconds, and a rotational correlation time τ_{rot}=10 nanoseconds in the free state and 1000 nanoseconds in the bound state. Figure 11 shows results for 0%, 25%, 50%, 75%, and 100% binding. The extent of binding of the labeled molecule can be determined quickly and sensitively by measuring Δ_ω and r_ω at a single suitable frequency (e.g., ~20 MHz for Δ_ω, and <~10 MHz for r_ω), and then reading off the extent of binding from an empirical calibration curve. Alternatively, binding could be determined using LDI and LRI, among others, if the binding is associated with a change in analyte lifetime.

Figure 12 shows how Δ_ω (Panel A) and r_ω (Panel B) depend on ω for a simple binding system in the presence of 50% background. Here, the background has a fundamental anisotropy r_0=0.3 a luminescence lifetime τ=1 nanosecond, and a rotational correlation time time τ_{rot}=0.1 nanosecond. These conditions correspond to compositions having a long-lifetime analyte and a short-lifetime background; the effective luminescence lifetime of the background usually is short, probably 0.1 to 10 nanoseconds. Unfortunately, a comparison of Figures 11 and 12 shows that there are no frequencies at which either Δ_ω or r_ω is unaffected by the background. This greatly diminishes the utility of Δ_ω or r_ω, especially because background varies from sample to sample, and so generally cannot be included in a calibration curve.

These shortcomings are addressed by the invention, which provides alternative functions that better discriminate between analyte and background, without requiring information from a blank and without requiring a determination of the lifetime or intensity of the background. Two such functions, denoted "sine" and "kappa" functions, are described below.

Sine function. The sine function, or Ψ_ω, is a ratio of the parallel and perpendicular AC intensities, weighted by the sines of the parallel and perpendicular phases, respectively.

$$\Psi_\omega = \frac{AC_\parallel \sin(\phi_{\parallel\omega})}{AC_\perp \sin(\phi_{\perp\omega})} \tag{27}$$

Ψ_ω may be shown to be a ratio of the sine Fourier transforms $N_{p\omega}$ of the intensity decays in associated parallel and perpendicular measurements. To see this, simple trigonometry and the relationship $\phi_{p\omega} = \tan^{-1}(N_{p\omega} / D_{p\omega})$ give

$$\sin(\phi_{p\omega}) = \frac{N_{p\omega}}{\sqrt{N_{p\omega}^2 + D_{p\omega}^2}} \tag{28}$$

Then, using Equation 28 defining Λ_ω gives

$$\Psi_\omega = \frac{AC_\parallel \sin(\phi_{\parallel\omega})}{AC_\perp \sin(\phi_{\perp\omega})} = \frac{\sqrt{N_{\parallel\omega}^2 + D_{\parallel\omega}^2}}{\sqrt{N_{\perp\omega}^2 + D_{\perp\omega}^2}} \frac{\sin(\phi_{\parallel\omega})}{\sin(\phi_{\perp\omega})} = \frac{N_{\parallel\omega}}{N_{\perp\omega}} \tag{29}$$

Figure 13 shows how Ψ_ω depends on ω for the system of Figures 11 and 12, in the presence of 0% (Panel A) and 50% (Panel B) background. Generally, the lower the frequency, the less Ψ_ω is affected by the (short-lifetime) background. In particular, below ω~10 MHz, Ψ_ω is much less affected by background than Δ_ω and r_ω. However, as ω becomes small, θ_p also becomes small, and measurement of the sine becomes imprecise. The optimum modulation frequency will be determined by a balance of these factors, among others.

The behavior of Ψ_ω for short-lived signals can be understood as follows. Assume that there are n molecular components, each with a single luminescence lifetime τ_i and a single rotational correlation time $\tau_{rot,i}$. The fraction of the steady-state luminescence intensity (no polarizers) contributed by each component is given by Equation 7. In the time domain, the anisotropy of each component is given by

$$r_i(t) = r_{oi} e^{-t/\theta_i} \tag{30}$$

Then by the standard relationships

$$I_{\parallel}(t) = \frac{1}{3} I(t)(1 + 2r_i(t)); \qquad I_{\perp}(t) = \frac{1}{3} I(t)(1 - r_i(t)) \tag{31}$$

Taking the sine Fourier transform gives

$$N_{\parallel\omega} = \frac{1}{3}\{\sum_i a_i \tau_i [L(\omega\tau_i) + 2r_{oi} \frac{\sigma_i}{\tau_i} L(\omega\sigma_i)]\} \tag{32}$$

$$N_{\perp\omega} = \frac{1}{3}\{\sum_i a_i \tau_i [L(\omega\tau_i) - r_{oi} \frac{\sigma_i}{\tau_i} L(\omega\sigma_i)]\} \tag{33}$$

Here, L(x)=x/(1+x^2). For |x|<<1, L(x)~x and L(0)=0. L(x) reaches a maximum value of ½ at x=1. For |x|>>1, L(x)~1/x, and L(∞)=0. The rotational correlation time enters the system only through

$$\sigma_i = \frac{\tau_i \theta_i}{\tau_i + \theta_i} \tag{34}$$

Because $\frac{1}{2}\min(\tau_i, \theta_i) \le \sigma_i < \min(\tau_i, \theta_i)$, σ always is smaller than either τ or σ. The ratio $\sigma_i / \tau_i = \theta_i / (\tau_i + \theta_i) < 1$. Ψ_ω can be formed by taking a ratios of the N's and recalling that $\alpha_i \tau_i = f_i \sum_j \alpha_j \tau_j$.

$$\Psi_\omega = \frac{N_{\parallel\omega}}{N_{\perp\omega}} = \frac{\sum_i f_i [L(\omega\tau_i) + 2r_{oi} \frac{\sigma_i}{\tau_i} L(\omega\sigma_i)]}{\sum_i f_i [L(\omega\tau_i) - r_{oi} \frac{\sigma_i}{\tau_i} L(\omega\sigma_i)]} \tag{35}$$

Here, the normalizing sum canceled out of all the terms.

Based on the behavior of L(x) for small x, Ψ_ω gives small weight to signals from short-lived species ($\omega\tau_i$ or $\omega\sigma_i$<<1), in comparison to signals for which $\omega\tau_i$ or $\omega\sigma_i$~1. Ψ_ω also gives small weight to the anisotropy contributions of long-lived components that have extremely short rotational correlation times (i.e., $\omega\sigma_i << 1$, $\sigma_i / \tau_i << 1$).

Kappa function. The kappa function, or K_ω, is a ratio involving the parallel and perpendicular AC intensities, weighted in part by the cosines of the parallel and perpendicular phases, respectively.

$$K_\omega = \frac{I_{\parallel} - AC_{\parallel} \cos\phi_{\parallel\omega} - (I_{\perp} - AC_{\perp} \cos\phi_{\perp\omega})}{I_{\parallel} + AC_{\parallel} \cos\phi_{\parallel\omega} + 2(I_{\perp} + AC_{\perp} \cos\phi_{\perp\omega})} \tag{36}$$

K_ω may be shown to be a ratio involving lifetime-discriminated intensities, as defined above, in associated parallel and perpendicular measurements.

$$K_\omega = \frac{LDI_\parallel - LDI_\perp}{LDI_\parallel + 2LDI_\perp} \quad (37)$$

Equation 37 is analogous to anisotropy, as may be seen by comparing Equation 37 for K_ω with Equation 2 for r.

Figure 14 shows how K_ω depends on ω for the system of Figured 11 and 12, in the presence of 0% (solid lines) and 90% (dashed lines) background. Results for K_ω are similar to results for Ψ_ω, except that K_ω may be less sensitive than Ψ_ω to frequency for low frequencies, and to binding for high binding. Neither the kappa nor the sine function depends on properties of the background, so neither function requires use of a blank or a determination of the lifetime or intensity of the background.

5. Reference Compounds

The apparatus, methods, and compositions of matter provided by the invention also can be used to correct for modifications in analyte signal from scattering, absorption, and other modulators, including background, through use of a reference compound. These modifications may affect intensity and polarization, among others.

The compositions of matter provided by the invention may include first and second luminophores having emission spectra that overlap significantly, but luminescence emissions that may be resolved using lifetime-resolved methods. The first and second luminophores may include an analyte and a reference compound. The analyte may be designed to participate in an assay, and the reference compound may be designed to participate in an assay, and the reference compound may be designed to be inert and constant from assay to assay.

The apparatus provided by the invention may include a stage, light source, detector, processor, and first and second optical relay structures. These components are substantially as described above, especially in supporting and inducing an emission from a composition, and in detecting and converting the

emission to a signal. The emission may include fluorescence or phosphorescence.

The processor may use information in the signal to determine the intensity of the light emitted by the analyte and the intensity of the light emitted by the reference compound. The analyte and reference compound have luminescence lifetimes that are resolvable by lifetime-resolved methods, so that the intensities of the analyte and reference compound may be determined using lifetime-resolved methods. These methods may include frequency-domain methods, such as those described above for distinguishing analyte and background.

In the presence of a signal modulator, such as scattering or absorption, the apparent intensity I_C' of light detected from a composition will equal the product of a transmission factor T and the true intensity I_C of the light emitted from the composition.

$$I_c' = T \cdot I_c \qquad (1)$$

The transmission factor may include contributions from changes in the excitation light and changes in the emission light. The transmission factor typically (but not always) will range from zero to one.

If the composition contains both an analyte and a reference compound, the apparent intensity of the composition will equal the product of the transmission factor and the sum of the true intensity I_A of the analyte and the true intensity I_R of the reference compound.

$$I_c' = T \cdot (I_a + I_r) \qquad (2)$$

The apparent intensity I_a' of the analyte will equal the apparent intensity of the composition minus the apparent intensity of the reference compound. Similarly, the apparent intensity I_r' of the reference compound will equal the apparent intensity of the composition minus the apparent intensity of the analyte.

These intensities may be computed using LDI or LRI methods, among others. For example, a typical experiment may include a short-lifetime analyte and a long-lifetime reference compound, although other combinations also may be used. In this case, the apparent intensity of the analyte may be calculated using Equation 14, where the reference compound effectively is treated as long-lifetime background.

$$I_a' = T \cdot I_a = T \cdot (I_c - I_r) = I_c' \cdot \left(1 - \frac{1 - X_c}{1 - X_r}\right) \quad (3)$$

Similarly, the apparent intensity of the reference compound may be calculated using Equation 13, where the analyte effectively is treated as short-lifetime background.

$$I_r' = T \cdot I_r = T \cdot (I_c - I_a) = I_c' \cdot \frac{1 - X_c}{1 - X_r} \quad (4)$$

The processor also uses information in the signal to calculate a quantity that expresses the intensity of the analyte as a function of the intensity of the reference compound. This quantity may be a ratio of the intensity of the analyte to the intensity of the reference compound, among others.

$$\frac{I_a}{I_r} = \frac{I_a'}{I_r'} = \frac{X_c - X_r}{1 - X_c} \quad (5)$$

Such a ratio is independent of the degree of modulation in the sample, and thus will be comparable for every sample in a family of samples, if for example every sample has the same concentration of reference compound.

The processor also is capable of discriminating between the light emitted by the analyte, the light emitted by a reference compound, and a background, if all three have different lifetimes, using the dual-frequency lifetime-resolved methods described above (e.g., Equation16).

The methods provided by the invention may include various steps, including (1) providing a composition that includes the analyte and a reference compound, (2) illuminating the composition, so that light is emitted by the analyte and reference compound, (3) detecting the light emitted by the analyte and reference compound and converting it to a signal, (4) processing the signal to determine the intensity of the light emitted by the analyte and the intensity of the light emitted by the reference compound, and (5) calculating a quantity that expresses the intensity of the analyte as a function of the intensity of the light emitted by the reference compound. The methods also may include additional or alternative steps. The methods may be practiced using the apparatus described above.

The invention may handle a variety of analytes, reference compounds, and backgrounds. Generally, the excitation and emission spectra of the

reference compound should be the same as the excitation and emission spectra of the analyte, so that the intensity of the reference compound will be modulated by the same amount as the intensity of the analyte. (Because the factors that modulate detection of luminescence are generally wavelength dependent, reference compounds having different spectra than the analyte provide only a partial solution, at best.) For optimal resolution, the lifetime of the reference compound should be significantly larger or significantly smaller than the lifetime of the analyte, and the lifetimes of the reference compound and analyte should be greater than the lifetime of the background. Also for optimal resolution, the specific lifetime of the background should be confined to a range. These conditions apply for most assays of commercial interest; for example, in most high-throughput assays, the background from the microplate and assay components is under 10 nanoseconds. These are preferred conditions; because the lifetime-resolved methods described above are so sensitive, the composition actually need include only a small amount of the reference compound (roughly 2% of the total intensity), and the lifetimes of analyte, reference compound, and background can be reasonably similar.

The reference compound may be associated with the composition using a variety of mechanisms. The reference compound may be associated with the composition directly, for example, by dissolving or suspending (e.g., as a micelle) the reference compound in the composition. The reference compound also may be associated with the composition indirectly, for example, by incorporating the reference compound into or onto beads, other carriers, or sample containers associated with the composition.

Associating the reference compound with beads or other carriers has a number of advantages. The carriers may be suspended in the composition or allowed to sink to the bottom of the sample container holding the composition. The carriers also may be attached to the walls or bottom of the sample container, for example, by chemical linkages such as biotin-streptavadin. The carriers also may be rendered magnetic, so that they may be pulled to one part of the sample container (e.g., a side or bottom) to permit the composition to be analyzed with and without the reference compound.

Associating the reference compound with the sample container also has a number of advantages. The reference compound may be layered onto the

surface of the sample container, or formed into the plastic or other material used to form the sample container. Such approaches eliminate the need to add the reference compound to the composition, and they may prevent the reference compound from interacting with components of the composition and affecting the associated assay.

6. Conclusions

The invention provides apparatus, methods, and compositions of matter for improving signal resolution in optical spectroscopy. These improvements may be obtained without using information from a blank, and/or without requiring a determination of the lifetime or intensity of the background. These improvements also may be obtained irrespective of whether a significant amount of the background is being detected by the detector at the same time that light emitted by the analyte is being detected. Consequently, the invention permits discrimination between analyte and background and/or other non-analyte emitters in measurements performed in a single sample container. The invention also permits light to be detected and analyzed continuously, so that signal is not wasted and data collection is not slowed.

Although the invention has been disclosed in preferred forms, the specific embodiments thereof as disclosed and illustrated herein are not to be considered in a limiting sense, because numerous variations are possible. Applicants regard the subject matter of their invention to include all novel and nonobvious combinations and subcombinations of the various elements, features, functions, and/or properties disclosed herein. No single feature, function, element or property of the disclosed embodiments is essential. The following claims define certain combinations and subcombinations of features, functions, elements, and/or properties that are regarded as novel and nonobvious. Other combinations and subcombinations may be claimed through amendment of the present claims or presentation of new claims in this or a related application. Such claims, whether they are broader, narrower, or equal in scope to the original claims, also are regarded as included within the subject matter of applicants' invention.

WE CLAIM:

1. An apparatus for detecting light emitted by an analyte in a composition, the apparatus comprising:

a stage for supporting the composition;

a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte may be induced to emit light;

a detector and a second optical relay structure that directs light from the composition toward the detector, so that light transmitted from the composition may be detected and converted to a signal; and

a processor that uses information in the signal to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to light from a non-analyte emitter, without requiring a determination of the lifetime or intensity of the light from the non-analyte emitter.

2. The apparatus of claim 1, wherein at least a portion of the non-analyte emitter comprises background which causes light that is not attributable to the analyte to be detected by the detector.

3. The apparatus of claim 1, wherein at least a portion of the non-analyte emitter comprises a reference compound, and wherein the processor calculates a quantity that expresses the intensity of the analyte as a function of the intensity of the reference compound.

4. The apparatus of claim 3, wherein the processor also may discriminate between a third portion of the signal that is attributable to a second non-analyte emitter comprising background.

5. The apparatus of claim 3, wherein the intensity of the reference compound is indicative of light absorption or scattering effects.

6. An apparatus for detecting light emitted by an analyte in a composition. the apparatus comprising:

a stage for supporting the composition;

a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte may be induced to emit light;

a detector and a second optical relay structure that directs light from the composition toward the detector, so that light transmitted from the composition may be detected and converted to a signal; and

a processor that uses information in the signal to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background, without requiring a determination of the lifetime or intensity of the background.

7. The apparatus of claim 6, wherein the processor discriminates between the first and second portions of the signal without requiring use of information obtained from a blank.

8. The apparatus of claim 6, wherein the processor discriminates between the first and second portions of the signal irrespective of whether a significant amount of the background is being detected by the detector at the same time that light emitted by the analyte is being detected.

9. The apparatus of claim 6, wherein the processor discriminates between the first and second portions of the signal to calculate the luminescence lifetime of the analyte.

10. The apparatus of claim 6, wherein the processor discriminates between the first and second portions of the signal to calculate the intensity of the light emitted by the analyte.

11. The apparatus of claim 6, the first optical relay structure including an excitation polarizer, the second optical relay structure including an emission polarizer, wherein the processor discriminates between the first and second portions of the signal to calculate the polarization of the light emitted by the analyte.

12. The apparatus of claim 11, the analyte including two populations distinguishable by rotational mobility, wherein the processor uses the polarization of the light emitted by the analyte to discriminate between a plurality of signal components, each signal component due to emission of light from a different population of analyte.

13. The apparatus of claim 6, wherein the processor uses information in the signal to discriminate in the frequency-domain between the first and second portions of the signal.

14. The apparatus of claim 13, wherein the information in the signal is frequency-domain information.

15. The apparatus of claim 13, wherein the information in the signal is time-domain information, and wherein the processor transforms the time-domain information into frequency-domain information.

16. The apparatus of claim 13, wherein the processor discriminates between the first and second portions of the signal using phase, modulation, or phase and modulation information.

17. The apparatus of claim 6, wherein the wavelength of the light emitted by the analyte is in the range 200-1000 nanometers.

18. The apparatus of claim 6, wherein the light emitted by the analyte includes at least one of fluorescence and phosphorescence.

19. The apparatus of claim 6, the analyte being a first analyte, wherein the background includes a second analyte.

20. The apparatus of claim 6, the composition including a reference compound, wherein both the analyte and the reference compound may be induced to emit light by the light source, and wherein the processor may use the signal to calculate a quantity that expresses the intensity of the analyte as a function of the intensity of the reference compound.

21. An apparatus for detecting light emitted by an analyte in a composition, the apparatus comprising:

a stage for supporting the composition;

a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte may be induced to emit light;

a detector and a second optical relay structure that directs light from the composition toward the detector, so that light transmitted from the composition may be detected and converted to a signal; and

a processor that uses information in the signal to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background without requiring use of information obtained from a blank, irrespective of whether a significant amount of the background is being detected by the detector at the same time that light emitted by the analyte is being detected.

22. The apparatus of claim 21, wherein the processor uses the information received from the detector to discriminate, in the frequency-domain, between the first and second portions of the signal.

23. The apparatus of claim 21, wherein the lifetime of the analyte is at least twice the effective lifetime of the background.

24. The apparatus of claim 21, wherein the lifetime of the analyte is no more than half the effective lifetime of the background.

25. The apparatus of claim 21, wherein the background is characterized by two effective lifetimes, one shorter than the analyte lifetime, one longer than the analyte lifetime.

26. The apparatus of claim 25, wherein the lifetime of the analyte is at least twice the effective lifetime of the shorter-lifetime background, and wherein the lifetime of the analyte is no more than half the effective lifetime-of the longer-lifetime background.

27. An apparatus for detecting light emitted by an analyte in a composition, the apparatus comprising:

a stage for supporting the composition;

a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte may be induced to emit light;

a detector and a second optical relay structure that directs light from the composition toward the detector, so that light transmitted from the composition may be detected and converted to a signal; and

a processor that uses information in the signal to discriminate, in the frequency-domain, between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background, without requiring a determination of the intensity of the background.

28. The apparatus of claim 27, wherein the processor can discriminate between the first and second portions of the signal using frequency-domain information corresponding to a single frequency.

29. The apparatus of claim 27, the background being characterized by two effective lifetimes, one shorter than the analyte lifetime, one longer than the analyte lifetime, wherein the processor can discriminate between the first and second portions of the signal using frequency-domain information corresponding to two frequencies.

30. An apparatus for detecting light emitted by an analyte in a composition, the apparatus comprising:

a stage for supporting the composition;

a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte may be induced to emit light;

a detector and a second optical relay structure that directs light from the composition toward the detector, so that light transmitted from the composition may be detected and converted to a signal; and

a processor that uses information in the signal to discriminate, in the frequency-domain between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background, without requiring use of information obtained from a blank.

31. An apparatus for detecting light emitted by an analyte in a composition, the apparatus comprising:

a stage for supporting the composition;

a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte may be induced to emit light;

a detector and a second optical relay structure that directs light from the composition toward the detector, so that the intensity of the light transmitted from the composition may be detected and converted to a signal; and

a processor that determines the intensity of the light emitted by the analyte by discriminating, in the frequency-domain, between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background.

32. The apparatus of claim 31, wherein the intensity is a steady-state intensity.

33. An apparatus for detecting light emitted by an analyte in a composition. the apparatus comprising:

a stage for supporting the composition, the composition having first and second populations of the analyte, the first and second populations having different polarizations;

a light source and a first optical relay structure having an excitation polarizer, wherein the first optical relay structure directs light from the light source through the excitation polarizer toward the composition, so that the analyte may be induced to emit light;

a detector and a second optical relay structure having an emission polarizer, wherein the second optical relay structure directs light emitted from the composition through the emission polarizer toward the detector, so that the polarization of the light transmitted from the composition may be detected and converted to a signal; and

a processor that uses information regarding the light transmitted from the composition to discriminate between the first and second populations, by calculating a quantity related to the relative fractions of molecules in the first and second populations, the quantity being insensitive to the presence of a background.

34. The apparatus of claim 33, wherein the processor is capable of discriminating between the background and light emitted by the first and second populations of analyte.

35. The apparatus of claim 33, the polarization depending on luminescence lifetime, wherein the processor is capable of discriminating between light emitted by the first population and light emitted by the second population based on a difference in the luminescence lifetimes of the first and second populations.

36. The apparatus of claim 33, the polarization depending on rotational mobility, wherein the processor is capable of discriminating between light emitted by the first population and light emitted by the second population based on a difference in the rotational mobilities of the first and second populations.

37. A method for detecting light emitted by an analyte in a composition, the method comprising:

illuminating the composition, so that light is emitted by the analyte;

detecting light transmitted from the composition and converting it to a signal;

processing the signal to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background, without requiring determination of the lifetime or intensity of the background.

38. The method of claim 37, wherein the processing step uses lifetime resolved methods.

39. The method of claim 37, wherein the processing step uses frequency-domain methods.

40. A method for detecting light emitted by an analyte in a composition, the method comprising:

illuminating the composition, so that light is emitted by the analyte;

detecting light transmitted from the composition and converting it to a signal;

processing the signal to discriminate between a first portion of the signal that is attributable to the light emitted by the analyte and a second portion of the signal that is attributable to a background, without using information obtained from a blank.

41. An apparatus for determining the intensity of light emitted by a luminescent analyte in a composition that includes the analyte and a luminescent reference compound, the apparatus comprising:

a stage for supporting the composition;

a light source and a first optical relay structure that directs light from the light source toward the composition, so that the analyte and reference compound may be induced to emit light;

a detector and a second optical relay structure that directs light from the composition toward the detector, so that light transmitted from the composition may be detected and converted to a signal; and

a processor that uses information in the signal to determine the intensity of light emitted from the analyte as a function of the intensity of light emitted from the reference compound by using lifetime-resolved methods.

42. The method of claim 41, wherein the processor calculates a ratio of the intensity of light emitted from the analyte to the intensity of light emitted from the reference compound.

43. The apparatus of claim 41, wherein the processor is capable of discriminating between a background and light emitted by the analyte and reference compound.

44. A method for determining the intensity of light emitted by a luminescent analyte in a composition that includes the analyte and a luminescent reference compound, the method comprising:

providing the composition;

illuminating the composition, so that light is emitted by the analyte and reference compound;

detecting the light emitted by the analyte and reference compound and converting it to a signal;

processing the signal to determine the intensity of light emitted from the analyte as a function of the intensity of light emitted from the reference compound by using lifetime-resolved methods.

45. The method of claim 44, wherein the processing step includes calculating a ratio of the intensity of light emitted from the analyte to the intensity of light emitted from the reference compound.

46. The method of claim 44, further comprising discriminating between a background and the light emitted by the analyte and reference compound.

47. The method of claim 44, wherein the emission spectrum of the analyte and the emission spectrum of the reference compound overlap significantly.

48. The method of claim 44, wherein the excitation spectrum of the analyte and the excitation spectrum of the reference compound overlap significantly.

49. The method of claim 44, wherein the lifetime-resolved methods include frequency-domain methods.

50. The apparatus of claim 44, wherein the light emitted by the analyte includes at least one of fluorescence and phosphorescence.

51. A composition of matter comprising first and second luminophores, wherein the emission spectra of the first and second luminophores overlap significantly, and wherein light emitted by the first luminophore is resolvable from light emitted by the second luminophore using lifetime-resolved methods.

52. The composition of claim 51, wherein the lifetime-resolved methods include frequency-domain methods.

53. The composition of claim 52, wherein the light emitted by the second luminophore is indicative of light absorbing or scattering effects.

54. The composition of claim 51, wherein the first luminophore is an analyte, and the second luminophore is a reference compound.

55. The composition of claim 51 further comprising reagents, wherein the first luminophore reacts to indicate the amount of a target substance, and the second luminophore is indicative of light absorbing or scattering effects independent of how much target substance is present.

56. A method for determining the rotational mobility of an analyte in a composition, the method comprising:

providing a composition that includes the analyte and a reference compound, the analyte and the reference compound being luminescent, the luminescence lifetimes of the analyte and reference compound being resolvable by lifetime-resolved methods;

illuminating the composition, so that light is emitted by the analyte and reference compound;

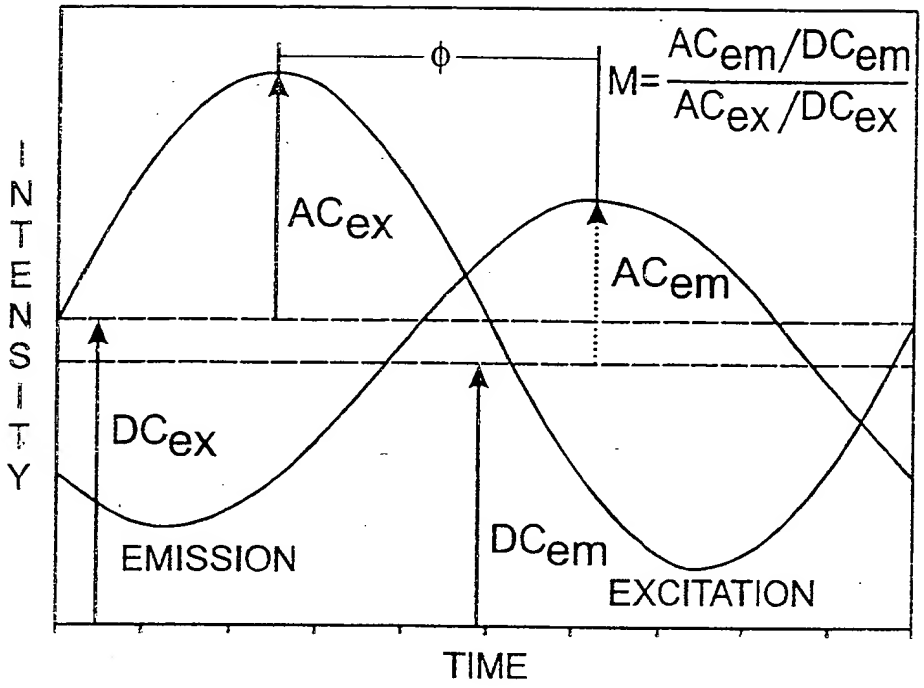
detecting the light emitted by the analyte and reference compound;

calculating the rotational mobility of the light emitted by the analyte and the rotational mobility of the light emitted by the reference compound, based on the light that they emit and their luminescence lifetimes; and

constructing a function that expresses the rotational mobility of the analyte relative to the rotational mobility of the reference compound.

57. The method of claim 56 further comprising calculating an amount of target substance in the composition based on the rotational mobility of the analyte.

Fig. 1


ϕ
M= ACem/DCem / ACex/DCex
ACex
ACem
INTENSITY
DCex
EMISSION
DCem
EXCITATION
TIME

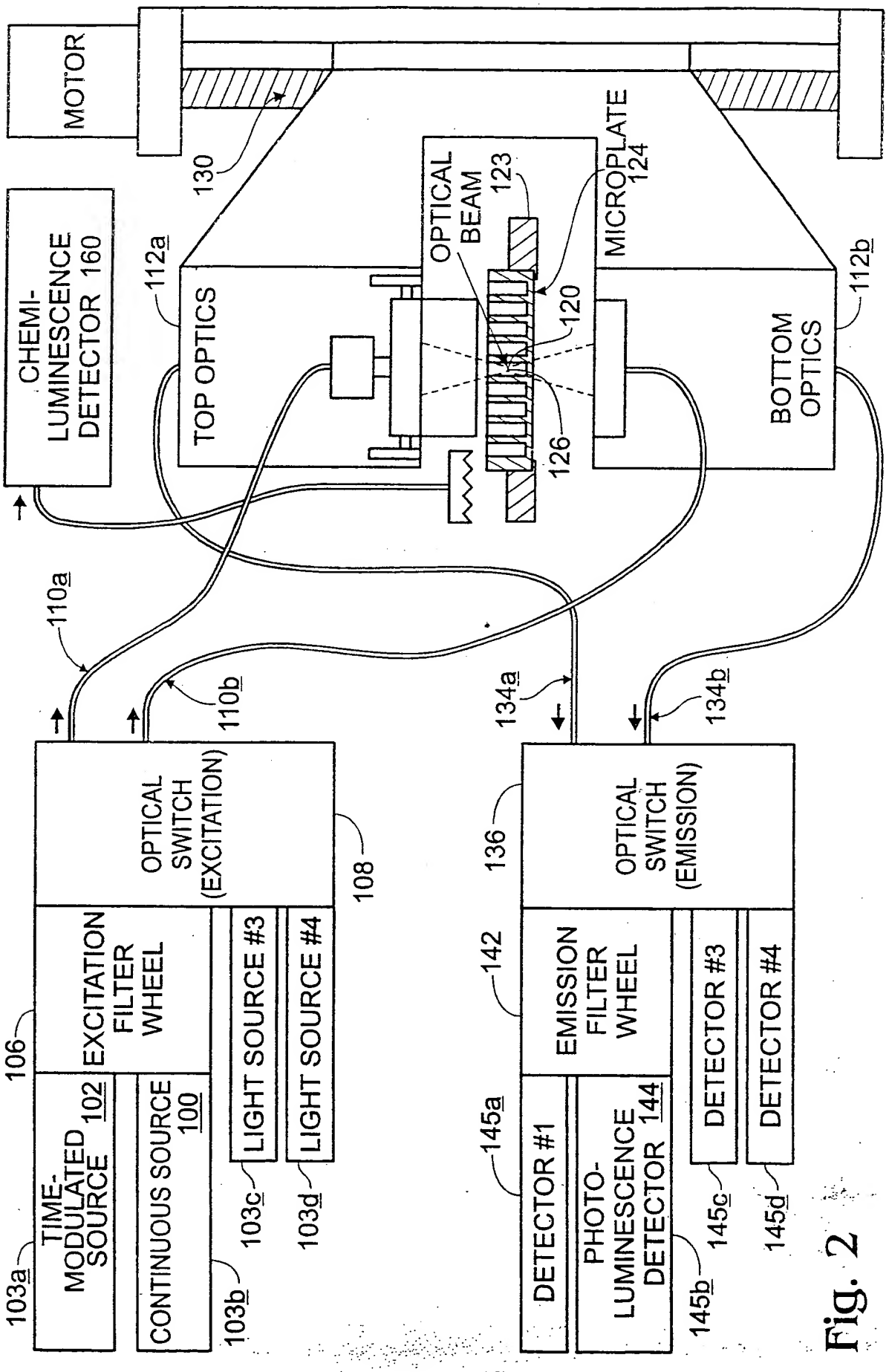

MOTOR
130
CHEMI-LUMINESCENCE DETECTOR 160
112a
TOP OPTICS
OPTICAL BEAM
123
MICROPLATE
124
120
126
BOTTOM OPTICS
112b
110a
110b
134a
134b
OPTICAL SWITCH (EXCITATION)
108
OPTICAL SWITCH (EMISSION)
136
106
EXCITATION FILTER WHEEL
LIGHT SOURCE #3
LIGHT SOURCE #4
142
EMISSION FILTER WHEEL
DETECTOR #3
DETECTOR #4
TIME-MODULATED SOURCE 102
CONTINUOUS SOURCE 100
DETECTOR #1
PHOTO-LUMINESCENCE DETECTOR 144
103a
103b
103c
103d
145a
145b
145c
145d

Fig. 2

Fig. 3

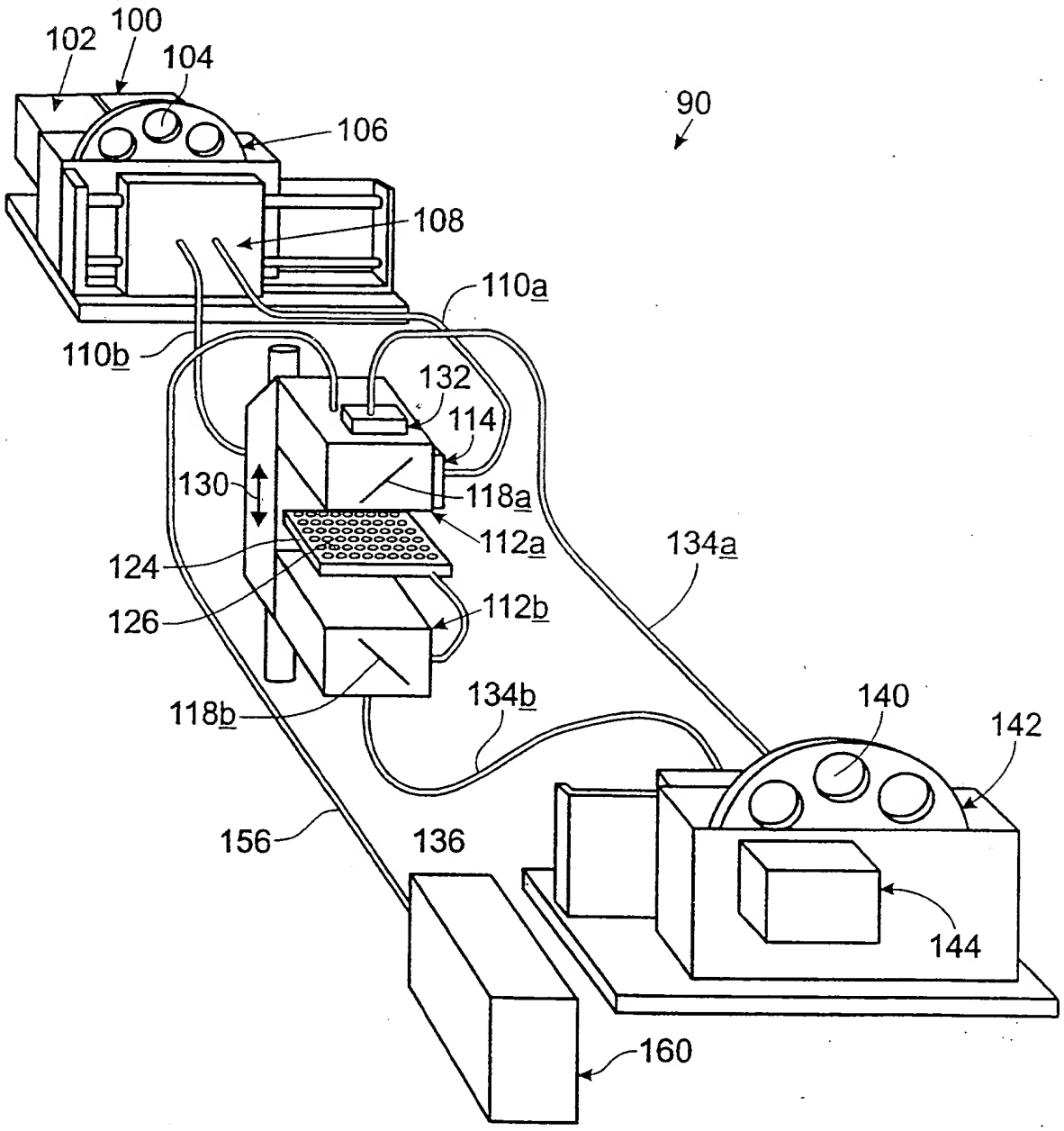
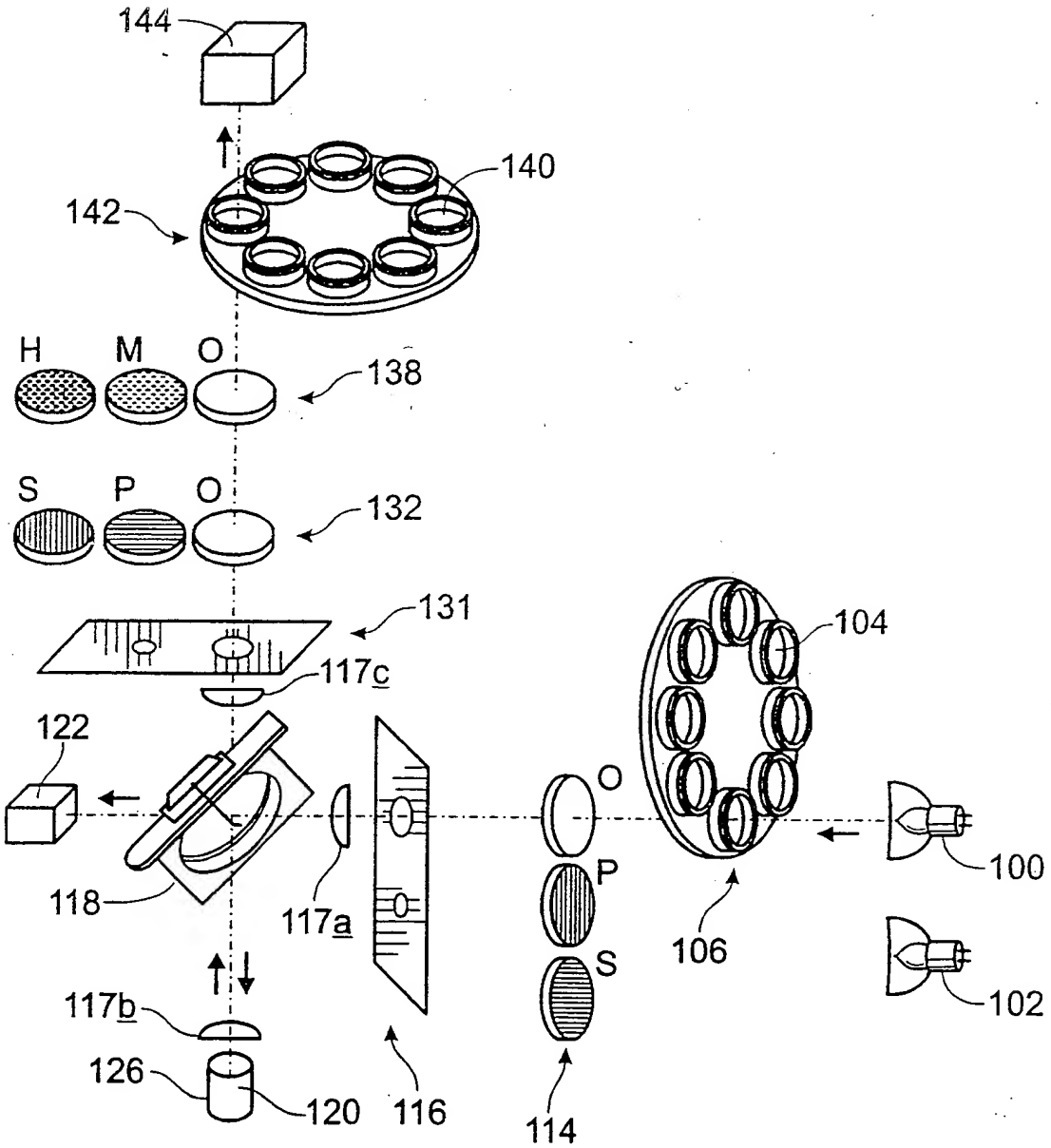

102
100
104
90
106
108
110a
110b
132
114
130
118a
112a
134a
124
126
112b
134b
140
142
118b
156
136
144
160

Fig. 4


144
140
142
H
M
O
138
S
P
O
132
131
117c
122
104
O
P
S
118
117a
106
100
102
117b
126
120
116
114

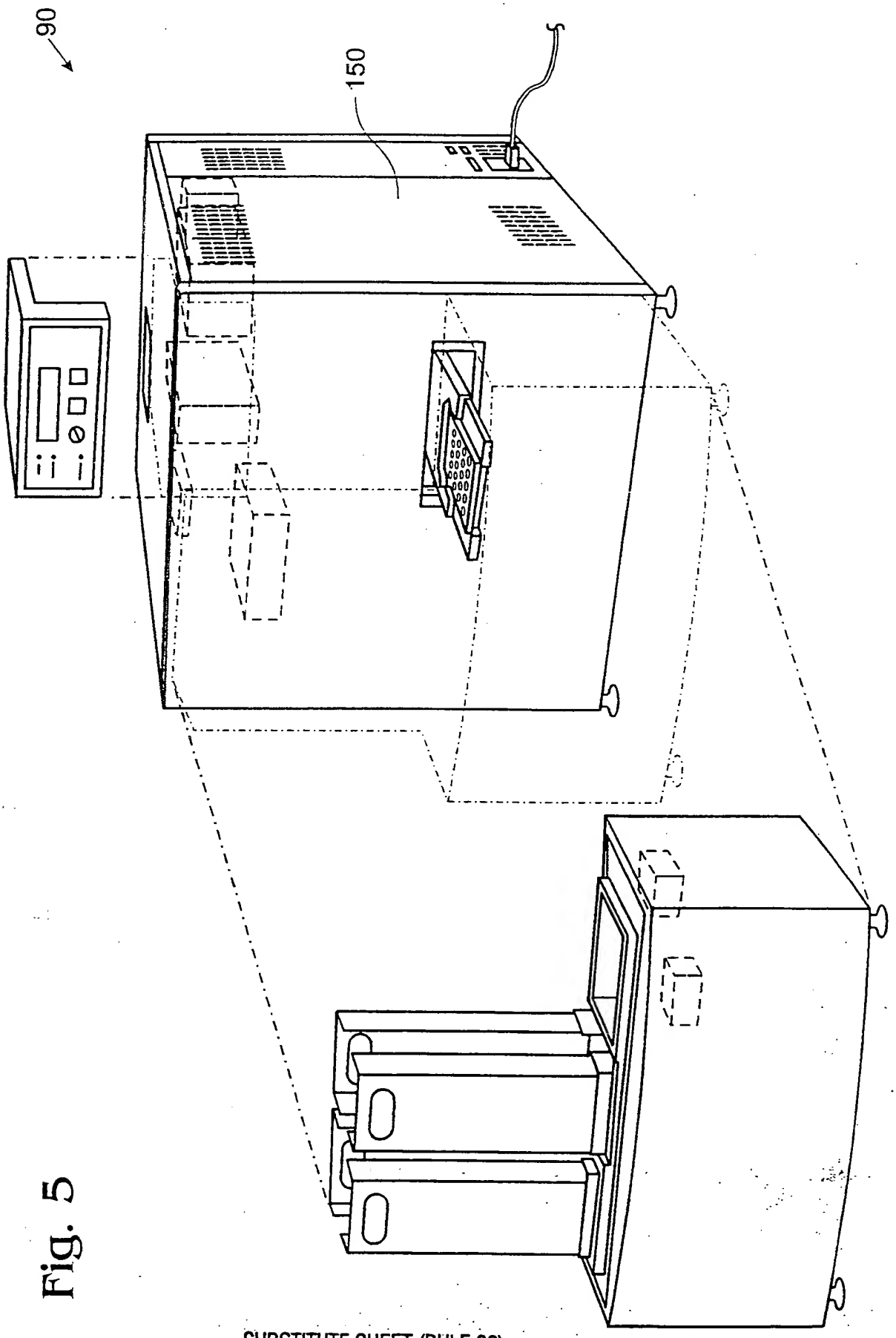


Fig. 5

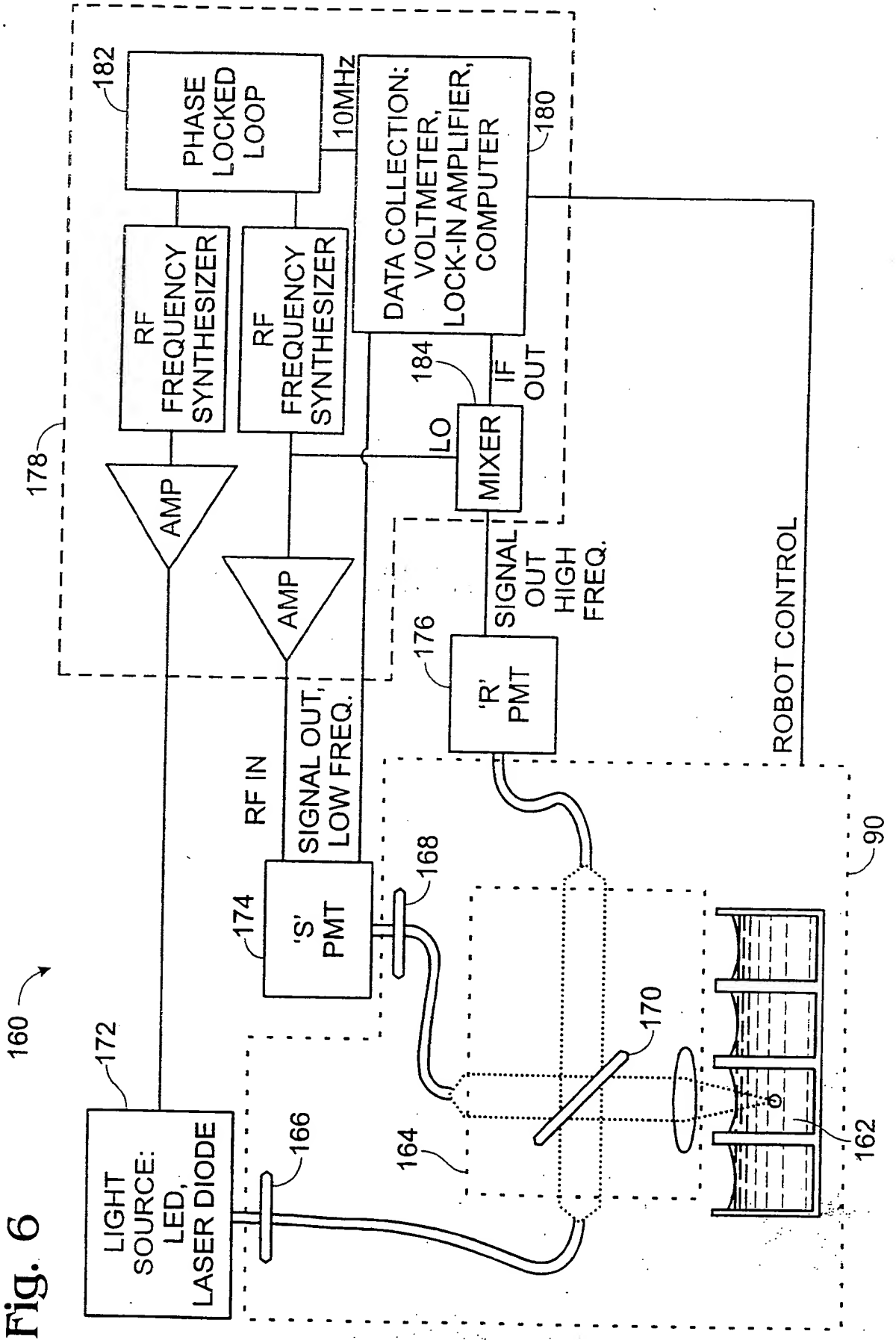

182
PHASE LOCKED LOOP
10MHz
DATA COLLECTION: VOLTMETER, LOCK-IN AMPLIFIER, COMPUTER
180
RF FREQUENCY SYNTHESIZER
RF FREQUENCY SYNTHESIZER
184
IF OUT
LO
MIXER
178
AMP
AMP
SIGNAL OUT HIGH FREQ.
ROBOT CONTROL
176
'R' PMT
RF IN
SIGNAL OUT, LOW FREQ.
90
168
174
'S' PMT
160
170
172
LIGHT SOURCE: LED, LASER DIODE
166
164
162

Fig. 6

Fig. 7

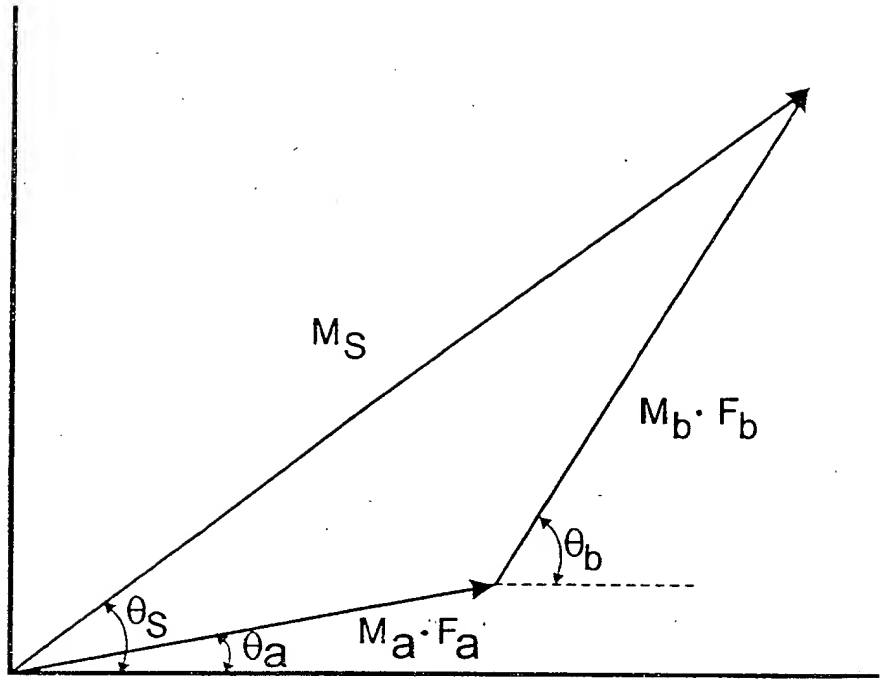

M_S
$M_b \cdot F_b$
θ_b
θ_S
θ_a
$M_a \cdot F_a$

Fig. 8

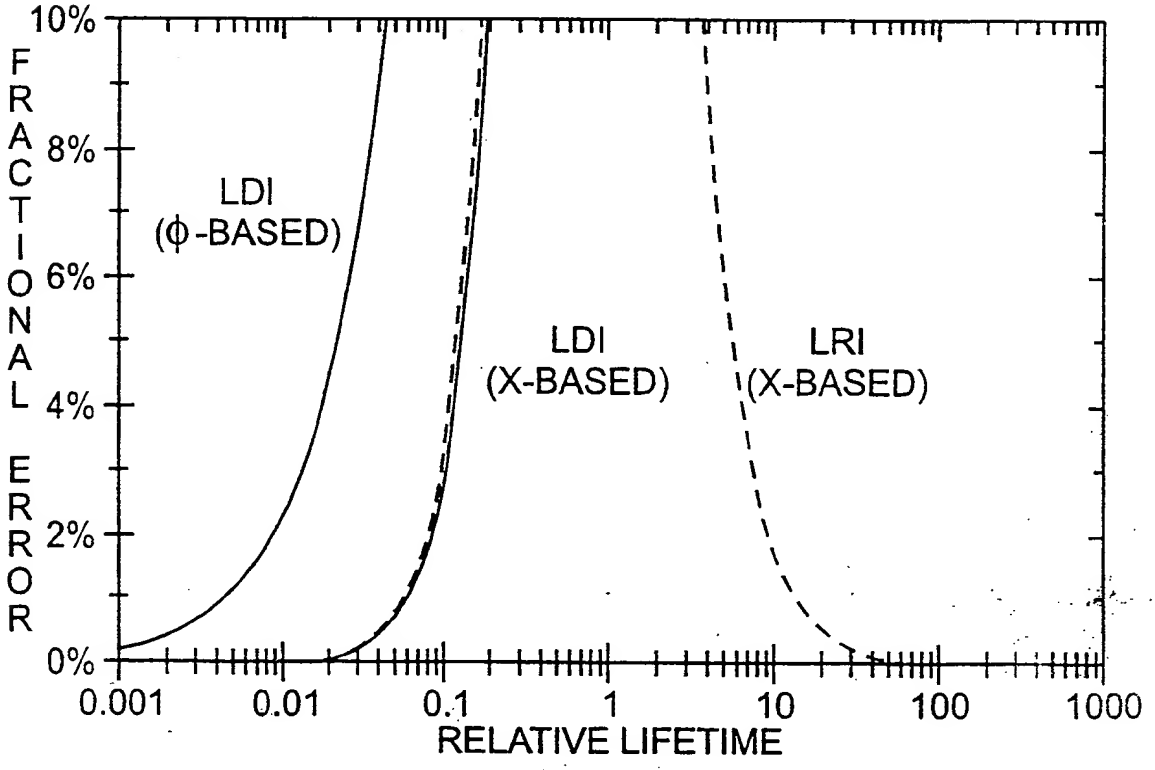

10%
8%
6%
4%
2%
0%
FRACTIONAL ERROR
LDI
(ϕ-BASED)
LDI
(X-BASED)
LRI
(X-BASED)
0.001
0.01
0.1
1
10
100
1000
RELATIVE LIFETIME

Fig. 9

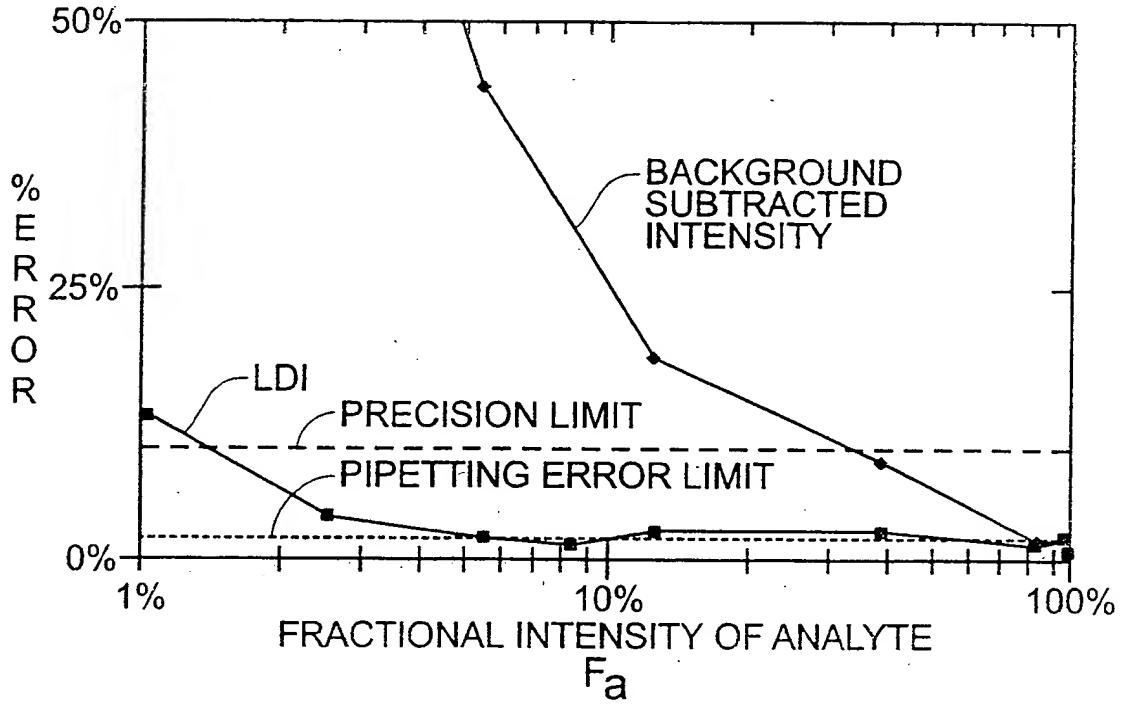

50%
25%
0%
% ERROR
BACKGROUND SUBTRACTED INTENSITY
LDI
PRECISION LIMIT
PIPETTING ERROR LIMIT
1%
10%
100%
FRACTIONAL INTENSITY OF ANALYTE
Fa

Fig. 10

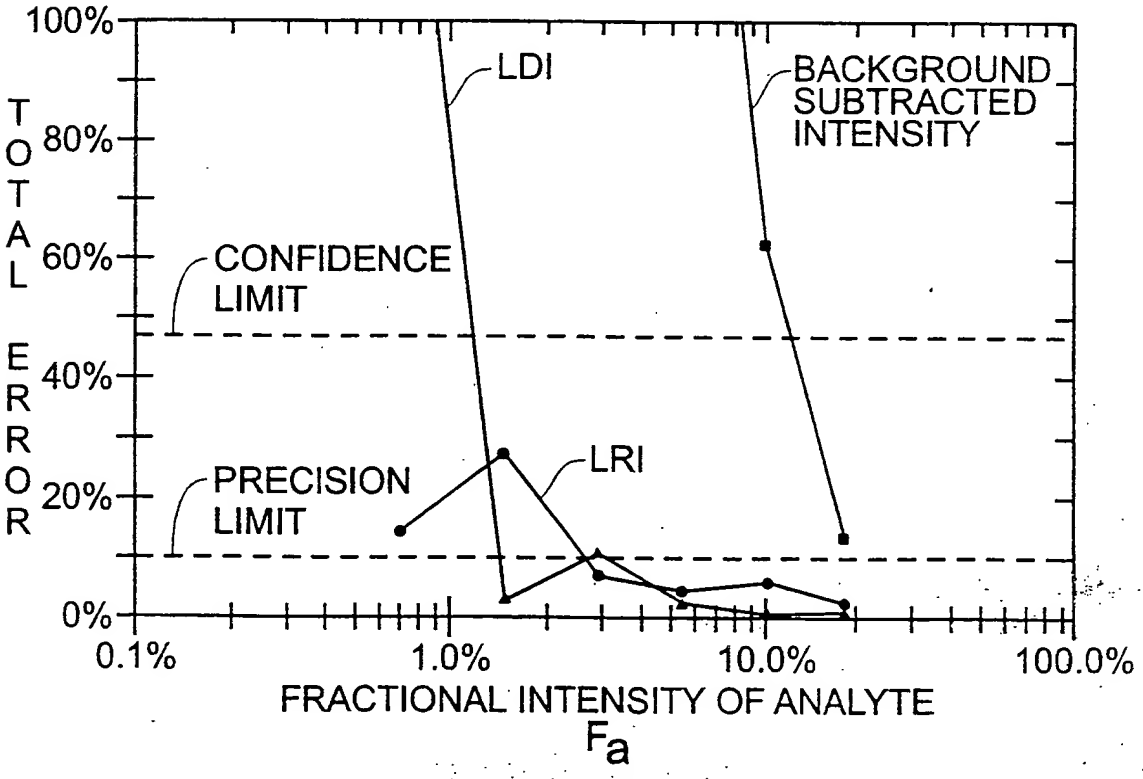

100%
80%
60%
40%
20%
0%
TOTAL ERROR
LDI
BACKGROUND SUBTRACTED INTENSITY
CONFIDENCE LIMIT
LRI
PRECISION LIMIT
0.1%
1.0%
10.0%
100.0%
FRACTIONAL INTENSITY OF ANALYTE
Fa

Fig. 11A

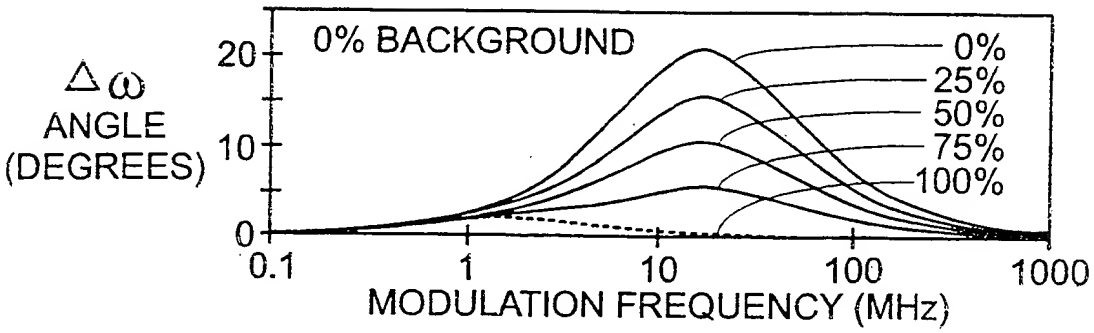


Fig. 11B

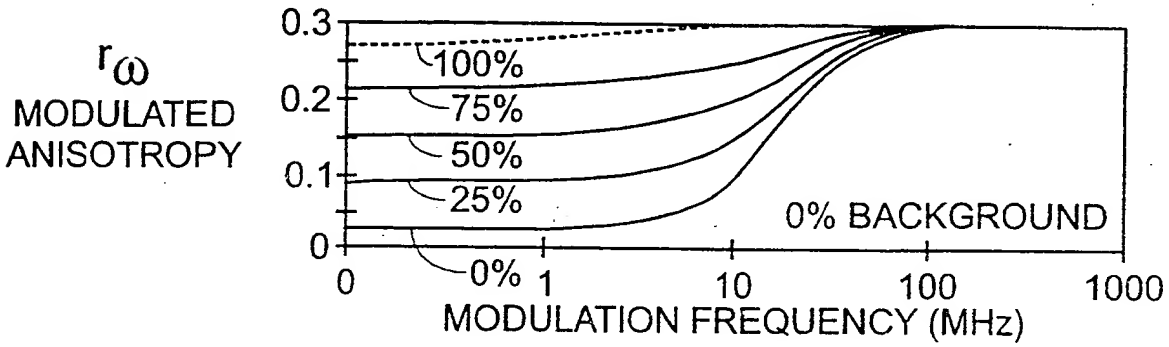


Fig. 12A

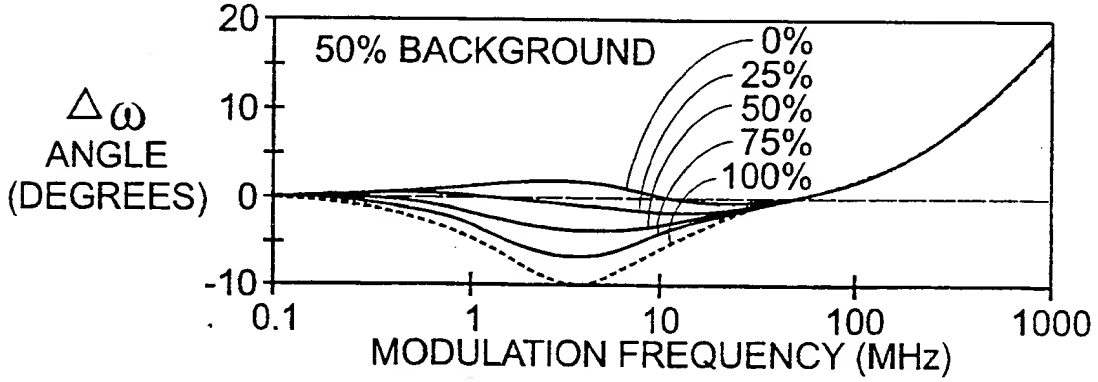


Fig. 12B

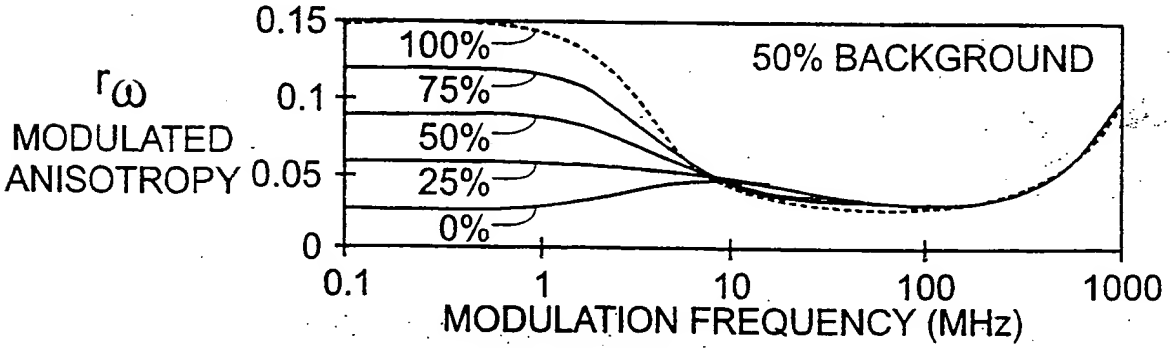


Fig. 13

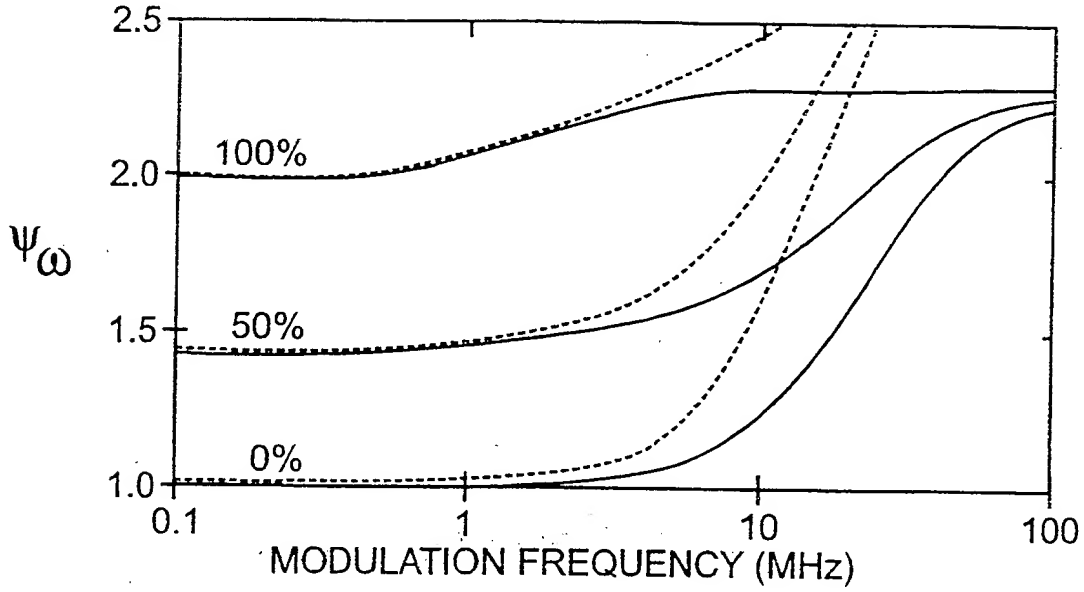
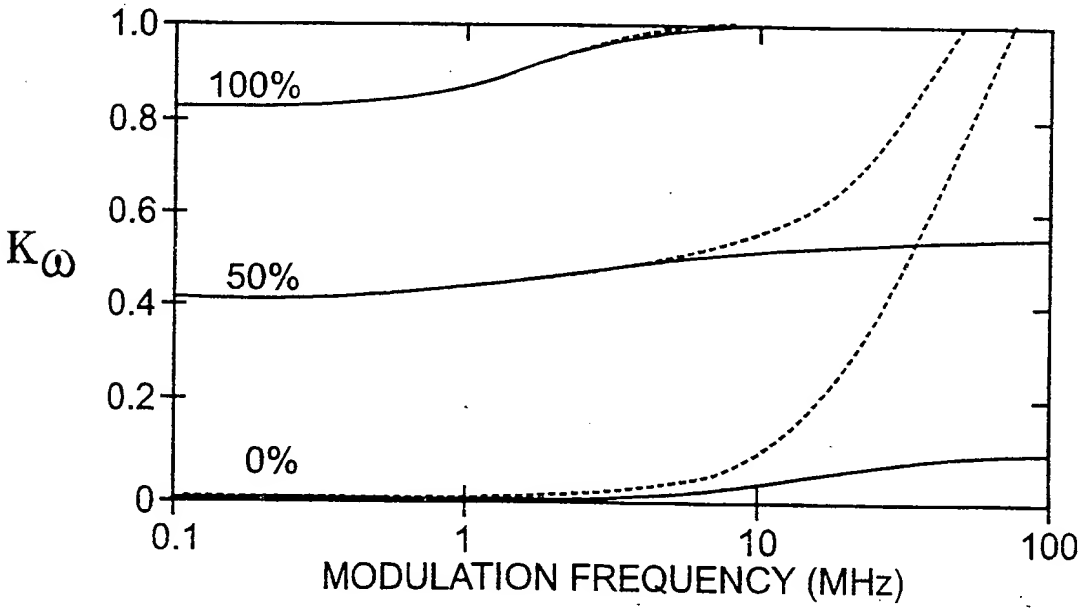

2.5
2.0
1.5
1.0
Ψ_ω
100%
50%
0%
0.1
1
10
100
MODULATION FREQUENCY (MHz)

Fig. 14


1.0
0.8
0.6
0.4
0.2
0
K_ω
100%
50%
0%
0.1
1
10
100
MODULATION FREQUENCY (MHz)